Effects of iso-alpha-acids on rumen fermentation and comparison of microbial populations between rumens and continuous culture fermenters

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ABSTRACT

Two experiments were conducted using dual-flow continuous culture fermenters to study rumen fermentation *in vitro*. The first experiment examined the effects of iso-alpha-acids from *Humulus lupulus* (Hops) extract on rumen fermentation. Iso-alpha-acid extract (IE) was provided to continuous culture fermenters to supply 0 (CON), 600 (LOW), 1200 (MED) or 1800 mg of IE/kg of diet DM/day. There was no effect ($P > 0.05$) of IE inclusion on DM, OM, NDF or ADF digestion (%). Volatile fatty acid (VFA) metabolism was not affected by IE treatment ($P > 0.05$), with total VFA concentrations of 105.5, 93.4, 87.9 and 103.6 mM for the CON, LOW, MED and HIGH treatments, respectively. Nitrogen metabolism was also not affected ($P > 0.05$) by IE level, with the CON, LOW MED, and HIGH treatments resulting in nitrogen concentrations of 7.4, 5.3, 7.6 and 6.8 mg N/dL of rumen fluid, respectively. No effects ($P > 0.05$) of treatment on fermenter pH were observed. It was concluded that administration of IE had no impact on fermentation by ruminal microbes maintained in continuous culture fermenters. Experiment 2 used 16S amplicon sequencing to compare microbial populations between the rumen of dairy cattle and continuous culture fermenters and to determine the temporal changes in microbial community during fermenter operation. Redundancy analysis (RDA) was performed to determine correlations between fermentation measurements based on microbial community. Correlations were also conducted to determine associations between prominent microbial families and fermentation measures from the *In vitro* system. Differences in microbial community were assessed using UniFrac metrics, Analysis of molecular variance (AMOVA) and analysis of similarity (ANOSIM) based on Bray-Curtis dissimilarity matrices. Differences in taxonomic composition of

different sample types were analyzed at kingdom, phylum, class, order and family taxonomic levels. Functional inferences were made by matching taxonomic data to KEGG Orthology terms using PICRUSt software, and analyzed based on sample type. Community profile did not differ ($P > 0.10$) between cows in either rumen or inoculum samples, but was different ($P < 0.05$) in fermenter samples. Microbial community within fermenters appeared to stabilize on day 7 of the experimental period according to AMOVA and ANOSIM analyses. *Bacteroidetes* and *Firmicutes* made up the two most abundant phyla in rumen, inoculum and fermenters and neither group was different ($P > 0.10$) based on sample type. *Proteobacteria*, *Tenericutes*, *Spirochaetes* and *Verrucomicrobia* were different ($P < 0.05$) between sample types. Rumen, inoculum and fermenters did not differ ($P > 0.10$) in relative abundance of *Prevotellaceae*, which was the most abundant family in all three samples. Abundant families that were different ($P < 0.05$) by sample type included *Succinivibrionaceae*, *Lachnospiraceae* and *Paraprevotellaceae*. PICRUSt predictions showed that amino acid metabolism, membrane transport, energy metabolism and cellular processes and signaling were different ($P < 0.05$) between sample types. Metabolism of carbohydrates, cofactors and vitamins, and lipids were not affected ($P > 0.10$) by sample type according to PICRUSt inferences.

Keywords: Rumen, Continuous Culture, Hops, Iso-alpha-acids, 16S Amplicon Sequencing, Functional Inferences

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INTRODUCTION

Understanding the complex symbiotic relationship between ruminant animals and their microbial inhabitants is imperative to maximize ruminant production. Ruminants have a unique adaption of an anaerobic pregastric fermentation chamber that harbors a diverse consortia of microbial species. These microbes are able to enzymatically degrade cellulosic plant materials that cannot be broken down by mammalian tissues, allowing the animal to utilize fibrous feedstuffs as a primary energy source. This adaptation provides tremendous benefit to the animal because cellulose represents the world's largest source of metabolizable carbon (Van Soest, 1994). Microbial fermentation yields volatile fatty acids (VFA) that are absorbed through the rumen epithelium and undergo gluconeogenesis and lipogenesis to provide energy to the animal. Herbivorous monogastrics can also synthesize and absorb VFA from microbial fermentation in the hindgut, however, the pregastric location of the rumen allows more efficient utilization of low-quality feed by providing high-quality protein from microbial cells. Rumen microbes are able to synthesize their own protein from non-protein nitrogen (NPN) sources such as urea and plant nucleic acids, converting NPN into protein that is metabolizable by the animal. In addition, the animal has the ability to recycle ammonia through concentration-dependent diffusion across the rumen epithelium and by incorporation of urea into saliva.

Similar to the synergism between ruminants and their microbial inhabitants, humans have a long history of interdependence with these unique animals. The ability of ruminants to transform plant components that are undegradable by mammalian enzymes into meat, milk, fiber and labor has long been recognized as a valuable component of human society. It is believed that humans hunted ruminants as a food source as early as

750,000 years ago (Church, 1988). Archeological evidence suggests that humans first began domesticating ruminants over 10,000 years ago, around the time when cultivation of crops was introduced (Cole and Ronning, 1974). The ability or ruminants to provide food and draft power without directly competing with humans for crops was a major factor in development of agrarian-based civilization. Further evidence from Neolithic farming sites suggest that during a period of climate hardship, the number of domestic ruminants drastically rose due to their adaptability to a wide range of range of climates (Balter, 2010). Because of their versatility, the use of ruminants as livestock has become widespread across the world.

Ruminants remain vital to the economies of both developed and developing countries. Human population growth, combined with an increase in demand for animal products in newly developed countries has put pressure on improvement of efficiency of livestock production. Since 1970, annual yearly meat and dairy consumption have increased by 5.1 and 3.6%, respectively (FAO, 2012). A common argument against the use of animals for food production is that pound for pound, grains are more efficiently utilized when they are fed directly to humans rather than to animals. Contrary to this belief, United States dairy cows have been estimated to be between 96 and 276% efficient at converting feed into milk protein on a humanly-consumable basis (Oltjen and Beckett, 1996). The ability of ruminants to utilize of nutrients that are unavailable to humans makes them an integral part of sustainable food production systems. Their utilization of humanly-indigestible feedstuffs is particularly valuable in the developing world. Their ability to convert grassland and crop byproducts from resource-poor land

into valuable meat and milk has been suggested as a tremendous opportunity to improve profitability of small farms in these areas (Delgado, 2005).

As the demand for animal products increases due to economic growth and development in the world, demands are placed on farmers and animal scientists to improve production efficiency of ruminants. In order to achieve this goal, it is critical to understand rumen ecology and function. Different rumen microbial species fill vastly different niches for nutrient utilization within the rumen. Slight modifications in the rumen microbiome can have tremendous impacts on the degradation and synthesis of nutrients within the rumen, resulting in major downstream effects on metabolism of the host and the productivity of the animal. Modulating the rumen microbial community can affect the synthesis of fermentation byproducts such as methane, hydrogen sulfide gas, and ammonia. These compounds all represent wasted nutrients to the animal and are associated with environmental pollution. While humans have been studying rumen microbiology since the 1800's, there is still much to be learned about the various roles microorganisms play in affecting the nutrition of the host animal. The dawn of new technologies like complex computer models, biological monitoring systems and constantly-improving genomic sequencing technologies, have and will continue to aid in improving the knowledge of rumen fermentation. Speed and scope of data collection by these technologies allows us to rapidly gather information to correlate rumen function to microbial populations. These improvements, along with improvements in genetic selection, animal husbandry and feed production will continue to maximize efficiency of ruminant production into the future. This review will characterize current knowledge

regarding rumen microbial ecology and fermentation, as well as feed additives with specific actions against rumen microbes.

RUMEN FERMENTATION

Anatomy and Physiology of the Ruminant Stomach

The most obvious anatomical peculiarity distinguishing ruminants from other mammals is their four-compartment stomach, consisting of the rumen, reticulum, omasum and abomasum. The rumen is the first compartment and functions as an anaerobic fermentation chamber with the ability to selectively retain feed particles for microbial degradation. Pillars of smooth muscle divide the rumen into four primary sacs, that serve to mix ruminal contents and separate them into solid, liquid and gas phases (Church, 1988). Digestion is aided by the ruminant animal's ability to regurgitate and remasticate ingested feed in a process called rumination. This process also aids in buffering rumen pH by increasing output of saliva, which contains several bicarbonate salts (Van Soest, 1994). A diverse host of microbial species within the rumen facilitates a wide variety of microbial processes, allowing for efficient use of feed nutrients. The rumen epithelium contains several transporters and aquaporins that allow absorption of fermentation end products such as VFA and ammonia, and directly transport these products to the liver for further metabolism (Stevens and Stettler, 1966; Houpt and Houpt, 1968). The rumen wall consists of a carpet of finger-like villi that serve to increase surface area and absorptive ability to maximize nutrient uptake (Graham and Simmons, 2005).

The second stomach compartment is the reticulum, which is an auxiliary pouch that is directly attached to the rumen, prompting the two compartments to collectively be referred to as the rumino-reticulum. The reticulum is primarily responsible for selective filtration of feed particles from the rumen to the remainder of the digestive tract (Church, 1988). A honeycomb-shape to the epithelium allows passage of small digested feed particles while retaining large particles for further digestion by microbes. Synchronized contractions of muscles surrounding the rumino-reticulum aid in mixing rumen contents, allowing inoculation of newly ingested feed. These contractions also aid in passage of feed into the large intestine, regurgitation of cud boluses for rumination and eructation of gas.

Feed leaves the reticulum through the omasal orifice at the base of the reticulum and enters into the omasum. The omasal epithelium consists of longitudinal folds that aid in absorption of water and residual volatile fatty acids. It is believed that the large folds trap feed particles and improve water and nutrient absorption (Smith, 1984). The final stomach compartment is the abomasum which is most similar to the stomach of monogastrics. Pepsin and hydrochloric acid are secreted from the glandular lining of the abomasum to breakdown feed particles and cause lysis of microbial cells from the rumen. Consistent with non-ruminants, mucin is secreted from the stomach lining to coat and protect the stomach from acid damage. At the posterior end of the abomasum, the pyloric sphincter regulates passage of digesta into the small intestine for further digestion and absorption (Church, 1988).

Rumen Nutrient Metabolism

Carbohydrate Metabolism

Rumen carbohydrate metabolism is defined by the ability of rumen microbes to degrade the complex cell wall compounds of feed through enzymatic action. The plant cell wall consists of a diverse range of polysaccharides including cellulose, mixed-linkage β-glucans, heteroglucans, glucuronarabinoxylans, and heteroxylans (Jung, 1997). Glycan and xylan bonds that are undegradable by mammalian enzymes can be degraded by various carbohydrases produced by rumen bacteria, protozoa and fungi. Fiber degradation is complicated by the presence of lignin, which is made of indigestible phenolic compounds that covalently crosslink with structural polysaccharides and is closely associated with hemicellulose (Sullivan, 1966; Van Soest, 1994). The degree of ruminal fiber degradation is customarily measured by the detergent fiber system (Van Soest et al., 1991). Neutral detergent fiber (NDF) is measured through the addition of a neutral detergent solution to a feedstuff, which removes sugars, starches and pectins, leaving only lignin, hemicellulose and cellulose. Acid detergent fiber (ADF) analysis uses a sulfuric acid solution to remove the remaining hemicellulose, leaving only lignin and cellulose which are strongly correlated with digestibility of the feedstuff (Van Soest, 1994). In addition, NDF digestibility (NDFd) measures the ability of NDF components to be broken down by rumen microbes, and is accepted as an accurate method to evaluate fiber digestibility (Oba and Allen, 1999; Goeser and Combs, 2009).

Fiber digestion in ruminants is determined by plant structure and composition, population densities of predominant fiber-digesting rumen microbes, microbial adherence

to the plant cell wall and animal factors influencing mastication, salivation, and digestion kinetics (Varga and Kolver, 1997). Plant cell wall components and degree of lignification vary greatly between different plant species. For example, legumes typically have a higher rate of digestion than grasses because of a lower total fiber content, however, digestibility of fiber is typically lower in legumes than in grasses due a greater degree of lignin crosslinking (Buxton and Redfearn, 1997). During plant growth, moisture and temperature can impact the quantity and digestibility of fiber within a forage, as will variety and maturity of the plant (Van Soest, 1994). There have been efforts to genetically select for grasses with lower lignin content. For example, brown midrib corn silage is a genetic hybrid with greater dry matter (DM), organic matter (OM), NDF and ADF digestibilities than traditional corn silage (Tine et al., 2001).

Concentrations and species of fibrolytic microorganisms also affect fiber degradation in the rumen. Factors including passage rate, pH, anti-nutritional compounds and available nitrogen impact the numbers of fibrolytic bacteria, protozoa and fungi in the rumen (Russell, 2002). Faster passage rate is associated with lower extent of fiber degradation due to fiber particles leaving the rumen at earlier stages of digestion and shorter time for microbial growth (Varga and Kolver, 1997). Rumen passage rate is impacted by feed intake and total structural carbohydrate present in the diet (Shaver et al., 1986). Acidic rumen conditions below pH 6.0 are well known to inhibit fiber degradation, whereas pH below 5.5 severely depresses the growth of fibrolytic microbes and subsequent fiber digestion (Hoover, 1986; Grant and Mertens, 1992). Polyphenolics such as tannins have been revealed to decrease activity of pectinase and cellulose in rumen fluid (McSweeney et al., 2001). Furthermore, growth of all rumen microorganisms

including fibrolytics is dependent on availability of true protein and nonprotein nitrogen. Addition of dipeptides and amino acids (AA) to *in vitro* rumen culture has demonstrated an increase digestibility of NDF (Yang, 2002). Maximal cellulose digestion occurs when rumen ammonia nitrogen (NH_3-N) concentrations reach approximately 43 mg/dl (Hoover, 1986). In order for non-starch polysaccharides within the cell wall to be digested, recalcitrant portions of the plant cell wall such as the cuticle and silica layers must be penetrated (Harbers et al., 1981) Although many rumen microorganisms are responsible for fiber degradation, only a small number are able to perform this essential process (Flint et al., 2008). Cellular adhesion to the plant cell wall is conducted by structures such as cellulosomes, fimbrial connections or glycocalyces (Krause et al., 2003). These structures bind to cell wall layers and etch pits into plant surfaces, allowing polysaccharases to enter and degrade fibrous compounds (Van Soest, 1994) Adherence of these structures is dictated by microbial age, cuticle protection, plant surface area available, rumen pH, rumen temperature and presence of oxygen (Miron et al., 2001).

In addition to structural polysaccharides, rumen microbes degrade non-structural carbohydrates (NSC) like sugars, starches and fructans. In contrast to fiber, NSC are more rapidly degraded in the rumen and serve as an immediate energy source for rumen microbes. Starch is a heterogeneous mixture of amylose and amylopectin and is primarily found in the floury endosperm of cereal grains (Huntington, 1997). Ruminal digestibility of starch is modified by species, variety and processing of the grain (Theurer, 1986; Philippeau et al., 1999). Storage proteins may also bind to ruminal starch, preventing its ability to be fully digested by microbial amylases. In corn, for example, prolamin within the kernel resists enzymatic degradation of starch, limiting its rate of digestion (Lopes et

al., 2009). While low rumen pH greatly inhibits cellulose, hemicellulose and pectin degradation, it appears to have little to no effect on amylolytic rumen microbes (Grant and Mertens, 1992). Fructans appear in high levels in temperate grasses and are easily broken down by rumen microbes (Morrison, 1979). Simple sugars are typically found at low amounts in forages because most are converted into polymers for storage (Van Soest, 1994). Monosaccharides, disaccharides, and oligosacchardies are rapidly fermented in the rumen, with limited simple sugars escaping the rumen into the lower GI tract (Russell, 2002).

Carbohydrate fermentation precipitates production of VFA by microbes. These short-chain fatty acids are responsible for the providing approximately 70% of the total metabolizable energy supply in ruminants (Bergman, 1990). Acetate is a two-carbon fatty acid that is produced when rates of rumen fermentation are slow, e.g., when high forage diets are fed to the animal. After absorption across the rumen epithelium, acetate is oxidized for energy or used for *de novo* lipogenesis (Hood et al., 1972). Fermentation of starch primarily yields the three-carbon VFA propionate, which helps the animal meet its glucose requirements by undergoing gluconeogenesis in the liver. Butyrate is produced at a lower rate than acetate and propionate, but plays an important role in the development and maintenance of the rumen epithelium (Plöger et al., 2012). Little is known about factors affecting butyrate production, but infusion of exogenous butyrate is acknowledged to stimulate growth of butyrate-producing bacteria (Li et al., 2012). In high forage diets, acetate, propionate, and butyrate are typically found in a 70:20:10 ratio, whereas this ratio shifts to approximately 50:30:10 when high concentrates diets are fed (Lyle et al., 1981; Sutton et al., 2003). Besides the three primary VFA, rumen

fermentation produces several branched-chain fatty acids which are important for the synthesis microbial protein (Oltjen et al., 1971). Lactic acid is also produced by rumen microbes, and can accumulate when rapidly fermentable diets are provided to the animal. Because lactate is a much stronger acid than VFA high lactate production can cause ruminal acidosis, leading to inhibited fiber digestion, decreased feed intake, laminitis and potentially death in the most extreme cases (Russell et al., 1992).

Protein Metabolism

Dietary nitrogen is important in ruminant diets to support the growth of rumen microbes and ultimately for maintenance and production of the animal. Nitrogen is supplied to the rumen from dietary true protein, NPN, endogenous secretions or by urea recycling (Bach et al., 2005). True protein in the diet is divided into rumen degradable protein (RDP) and rumen undegradable protein (RUP) fractions. RDP represents dietary true protein that is broken down by rumen microbes into short peptides and AA, which can be directly incorporated into microbial protein or deaminated into free ammonia and carbon skeletons (Russell et al., 1992). The remaining RUP leaves the rumen undegraded and undergoes gastric digestion in the lower GI tract. Degradability of protein sources is highly variable within and between feedstuffs and depends greatly on feed processing (Kleinschmit et al., 2007; Larson and Stern, 2013, *unpublished data*). Compared to forages and grains, animal-derived products such as bloodmeal and fishmeal tend to have higher total protein, and a higher percent of RUP (Stern et al., 1994). Kleinschmit et al. (2007) found that RUP of distiller's dried grains (DDG) from different processing plants varied from 61.3% to 70.2%. The amount of proteolytic bacterial species also effects rate of protein degradation, but the dynamics of proteolytic species growth is poorly described

(Bach et al., 2005). In the future, high-throughput metagenomic sequencing has potential to provide insight into this relationship (Firkins et al., 2007, 2008).

Rumen microbes are responsible for supplying 60 – 85% of total metabolizable protein supplied to the small intestine of the animal (Storm and Ørskov, 1983). Microbes rely on nitrogen from RDP and NPN sources such as urea, ammonia, DNA and RNA, for microbial protein synthesis (MPS). Ammonia-N represents the most important nitrogen source for rumen microbes. Dairy cattle have to survived and maintained a reasonable level of milk production when fed diets devoid of true protein (Virtanen, 1966). Optimal ruminal ammonia concentrations for microbial fermentation have been established to be between 2 and 5 mg/dL (Satter and Slyter, 1974; Satter and Roffler, 1975; Slyter et al., 1979). However, bacterial growth in pure and mixed cultures increases with addition of peptides and AA (Cotta and Russell, 1982; Bach et al., 2005). The animal is also able to provide additional nitrogen to the rumen via urea recycling from diffusion across the rumen epithelium or through salivary secretion (Reynolds and Kristensen, 2008). Urea recycling across the rumen wall is facilitated by pH and ammonia concentration-dependent urea transporters (UT-B1, UT-B2) and possibly aquaporins in the cellular membrane of papillae (Røjen et al., 2011; Lu et al., 2014; Walpole et al., 2015). Urease produced by ruminal bacteria converts urea to ammonia, which is available for assimilation into microbial protein (Cook, 1976).

Because microbial protein makes up the majority of metabolizable protein available to the ruminant, the efficiency of microbial protein synthesis (EMPS) is an important factor in determining the animal's productivity. Several factors influence EMPS in the rumen, most importantly the synchrony of available of nitrogen and energy.

The importance of matching of energy and nitrogen availability has long been known to be an important factor in maximizing microbial protein outflows. Sinclair et al. (1995) demonstrated an 11 to 20% increase in MPS when cows were fed a diet designed to synchronize available nitrogen and energy. Because coordinating the availability of these two nutrients is so important, many ration formulation models for dairy cattle place their considerable focus on synchronizing them (Sniffen et al., 1992; Tamminga et al., 1994; NRC, 2001; Tedeschi et al., 2007; Sauvant and Nozière, 2013). Solids and liquids dilution rates also affect EMPS. *In vitro* continuous culture experiments have determined a positive correlation between dilution rates of both solid and liquid fraction of digesta, and EMPS (Isaacson et al., 1975; Hoover et al., 1984; Shriver et al., 1986; Schadt et al., 1999). Furthermore, EMPS is reliant on the abundance of sulfur, branched-chain volatile fatty acids and trace minerals because these nutrients are essential for bacterial synthesis of amino acids (Stern et al., 1994). Historically, the amount of microbial protein available to the animal has been measured by sampling from duodenal cannulas (Zinn and Owens, 1986; Clark et al., 1992). However, due to lysis of protozoa by hydrochloric acid in the abomasum, contributions of protozoa to microbial protein are often underrepresented using this technique (Sylvester et al., 2005). To avoid hydrolysis of protozoa, recent measurements of microbial protein have been derived by sampling digesta from the omasum (Broderick et al., 2010).

Microbial protein and RUP which leave the rumen and enter the lower GI tract are collectively called metabolizable protein (MP). Animals have no specific requirement for MP, but rather have requirements for individual amino acids. The availability of certain amino acids is limiting for the synthesis of protein involved in growth, production,

muscle protein turnover and biological functions such as hormone signaling and enzymatic catalysis. Specific amino acids are more limiting for than others, and proper amino acid balance is important to maximize efficiency of nutrient utilization by the animal (Schwab, 1996). Providing proper amino acid balance to the small intestine is difficult because deamination in the rumen changes the ratios of individual amino acids (Clark et al., 1992). This problem can be addressed by feeding high RUP feedstuffs with appropriate amino acid balances or by supplementation of protected amino acids (Mabjeesh et al., 1996; Boucher et al., 2009). Amino acids may be protected from ruminal degradation using a fat-amino-acid matrix or by encapsulation with a fat polymer (Schwab, 1996). Protected lysine, methionine, and histidine have all improved production response in lactating dairy cows (Socha et al., 2005; Giallongo et al., 2015).

Modeling Rumen Fermentation *In Vitro*

Several methods have been used to measure fermentation by rumen microbes *in vitro*. *In vivo* rumen fermentation studies are highly time and cost-intensive and generate error associated with inherent animal variation and digestive markers (Stern et al., 1997). *In vitro* systems conversely provides measures of rumen fermentation more in a more rapid, less expensive, and more controlled manner. In addition, *in vitro* techniques allow for administration of feed additives at levels potentially toxic to the animal (Hristov et al., 2012). Rumen microbes are very sensitive to environmental conditions and substrate. Therefore it is important for any rumen *in vitro* system to mimic the true rumen environment as closely as possible (Van Soest, 1994). This requires maintenance of an anaerobic environment, provision of an adequate amount of buffer solution, removal of

fermentation products, agitation of inoculum and maintenance of fermentation temperature similar to the physiological temperature of the animal.

In vitro batch culture fermentation can be used to provide a rapid measure of rumen fermentation during a limited time period. While these methods are not sustainable for measurement of long-term effects due to a lack of fermentation end-product removal, they can adequately compare *in vivo* digestion between feedstuffs. Tilley and Terry (1963) developed a two-stage technique involving the incubation of forage crops within a rumen liquor-buffer mixture followed by hydrolysis with a commercially available pepsin isolate. This technique has endured several modifications including differential dilutions of rumen fluid and artificial saliva, (Richards et al., 1995), tighter pH regulation (Grant and Mertens, 1992), and inclusion of particle-associated bacteria to strained rumen fluid inoculum (Craig et al., 1984). The first stage of this technique has been adapted to create an *in vitro* gas production technique that uses multiple regression analysis to calculate metabolizable energy content of feedstuff using the volume of gas produced when the feed has been inoculated with a rumen fluid culture. (Menke et al., 1979; Menke and Steingass, 1988). Because rumen fermentation produces CO_2, CH_4, and trace amounts of H_2, measurement of the total gas produced can predict the rate of digestion of a particular feedstuff (Menke et al., 1979). This technique has also been adapted to calculate gas volume based on pressure measured through computerized sensors (Pell and Schofield, 1993). However, inferences from the Tilley-Terry technique are limited because the amount of gas produced varies with the molar proportions of VFA produced. Several reviews have proposed mathematical approaches to solve this issue, with the most well-accepted approach using a dual-pool logistic equation and a single lag value to best

describe the relationship between cellulose digestion and gas production (Schofield et al., 1994). *In vitro* gas production methods have also been applied to the ethanol industry as a measure cellulosic biomass fermentability (Weimer et al., 2008).

Other methods involve purified enzymes to determine the extent of digestibility of feed components. These techniques have the advantage of determining digestibility completely independent of the animal, unlike batch and continuous culture techniques which rely on rumen fluid as a source of microbes, requiring animals as inoculum donors. Because of inherent variation in the microbial population of rumen fluid donors, eliminating the use of animals allows for a decrease in variation within and between trials. Attempts to predict the degradation of crude protein (CP), starch and cellulose in ruminant feeds have been made using purified enzymes. Kohn and Allen (1995) extracted proteolytic enzymes from rumen microbes using butanol and acetone and found the enzymatic mixture exhibited 62% of the proteolytic activity compared with complete rumen fluid. The use of α-amylase from *Bacillus subtilis* cultures was revealed to poorly represent ruminal starch degradation (Cone, 1991). Fungal cellulase produced a correlation between 0.93 and 0.98 using the Tilley and Terry technique for predicting the fiber digestibility of forage samples (Marten et al., 1988). Lila et al. (1986) combined amylolytic, cellulolytic and proteolytic enzymes and found low correlation between 0.11 and 0.49 compared to *in vivo* forage digestibility. Unfortunately, these methods are limited in their capacity to accurately describe *in vivo* feedstuff degradation due to their incomplete enzymatic activity compared with the rumen (Stern et al., 1997). This problem is exacerbated when nonruminal enzymes are used because they possess

different specific activities than enzymes produced by rumen microbes (Mahadevan et al., 1987).

Continuous-culture (CC) rumen fermenters may also be used to model rumen fermentation. These systems are able to remove fermentation end-products, allowing them to maintain a stable fermentation for a longer duration than batch culture (Hristov et al., 2012). Alder et al. (1958) developed a CC system that could maintain rumen culture for up to 10 hours using a protein-free diet. Stewart et al. (1961) improved upon this system to sustain a longer culture with more diverse substrate by constantly adding substrate and removing product through an electronic solenoid valve system. The values obtained for VFA and many bacterial populations were comparable to *in vivo* values, however the culture was only able to be maintained for a total of 24 hours due to frequent mechanical failure (Stewart et al., 1961). A system developed by Slyter et al. (1964) was able to maintain a steady state fermentation for 7 days with VFA and methane production values similar to *in vivo*. This system was able to preserve a stable bacterial population with VFA concentrations similar to *in vivo* values, but was unable to maintain a relevant population of protozoa (Slyter et al., 1967).

Hoover et al. (1976b) attempted to preserve protozoal populations by developing a CC system with differential solid and liquid output flow rates. This system was able to harbor protozoa at a level between 6.7×10^4 and 13.4×10^4 cells per mL. Although the protozoal population was lower than biological levels, they were much higher than observed with Slyter's system (Slyter et al., 1967; Hoover et al., 1976). The system was also able to accurately estimate diet effects on NDF and ADF digestion as well as VFA concentrations compared to *in vivo* (Hoover et al., 1976). Hoover's system was modified

by Hannah et al. (1986) to lessen fermentation volume, use N_2 gas to continuously purge the culture of O_2 and utilize a coaxial heat exchange unit to maintain fermenter temperature (Figure 1.1). This system was able provide a reasonable estimate of rumen fermentation with similar true OM, CP and AA digestion to *in vivo* observations (Hannah et al., 1986).

A long-term rumen simulation technique (RUSITEC) was developed by Czerkawski and Breckenridge (1977) as an alternative to other continuous-culture systems. This apparatus contains a total of four 1 L vessels secured to a base in a water bath maintained at 39°C. Nylon bags containing feed are placed within a polyethylene container and suspended in a reaction vessel containing rumen fluid inoculum. The polyethylene container is agitated within the reaction vessel using an electric motor to simulate the mixing of the rumen. Unlike single-vessel continuous culture systems, reaction vessels are gas tight and no gas is bubbled into the rumen fluid. One disadvantage of the RUSITEC system is that solid and liquid dilution rates are markedly lower compared with other continuous culture systems. Compared to non-RUSITEC continuous culture and *in vivo* measurements, NDF digestibility was lower with RUSITEC, which may be attributed to a decrease in fibrolytic bacteria within the nylon bags (Hristov et al., 2012).

Attempts have been made to model rumen microbial ecology within continuous culture fermentation systems. Mansfield et al (1995) used microbial culture to compare bacterial and protozoal populations in the continuous culture fermenters with rumen and duodenal cannulated lactating dairy cows. This experiment found significantly fewer protozoa within the CC fermenters than *in vivo*, including a complete disappearance of

Holotrich species (Mansfield et al., 1995). These results are similar to previous findings and are likely credited a to longer generation interval for protozoa and the inability for them to sequester themselves within the homogeneous conditions of the fermenter (Hoover et al., 1976a). Reciprocally, total bacterial numbers were greater *in vitro* than *in vivo*, probably due to less substrate competition with protozoa in the fermenters. Results are consistent with several *in vivo* studies which demonstrated that rumen defaunation increases bacterial numbers (Orpin and Letcher, 1984; Newbold and Hillman, 1990; Mosoni et al., 2011). Amylolytic bacterial concentrations were similar between natural rumens and fermenters, but the percentage of amylolytic bacteria was greater *in vivo*. The experiment did find similar concentrations of proteolytic bacteria and fungi between the rumen and CC fermenters (Mansfield et al., 1995). Zeimer et al. (2000) made similar comparisons between microbial populations of rumen and CC fermenter samples using 16S rRNA oligonucleotide probes. Their study found that the fermenter system maintained similar proportions of total *Fibrobacter succinogenes* and *F. succinogenes* subgroup 3 as the rumen after 240 h operation. Moreover, similar relative abundances of archaea populations were also noted between the rumen and CC fermenters. Consistent with Mansfield et al. (1995), an increase in relative proportions of bacteria and a reciprocal decrease in relative proportions of protozoa was observed.

RUMEN MICROBIAL ECOLOGY

Rumen Microbial Populations

The rumen ecosystem is concurrently stable and dynamic. The anaerobic environment and limited pH fluctuation permits the growth of a limited array of

microorganisms. These steady conditions are maintained by constant addition of substrate in the form of carbohydrates, amino acids and minerals which are required for microbial growth, and the removal of their end-products (VFA, microbial protein, ammonia). These conditions prevent contamination of the rumen by outside invaders, despite the fact that millions of foreign microorganisms encounter the rumen daily through feed, water, air and soil. Because of continuous digesta removal, growth rates of rumen microorganisms must be sufficient to establish a population despite the turnover of rumen contents (Stewart et al., 1997). While rumen conditions remain relatively constant in the long-term, microbes are able to adapt to short-term fluctuations in nutrient availability. Adaptability results from the diverse assortment of organisms that are able to survive within the limited range of rumen environmental conditions. As ruminants evolved to feed on a wide-range of plant materials their microbial inhabitants consequently co-evolved to develop the unique metabolic functions necessary to degrade the substrates they encountered (Morgavi et al., 2012). It has been estimated that as many as 16,000 bacterial taxa could reside within the rumen (Edwards et al., 2004). This estimate does not even consider the diversity provided by protozoa, archaea, fungi, and viruses. These groups distinctively affect rumen fermentation and confer different metabolic capabilities.

Bacteria

Bacteria make up the largest and most diverse population in the rumen, comprising approximately 95% of the total microbiota (Jami and Mizrahi, 2012a). The number of total rumen bacteria has been estimated at up to 10^{11} cells/mL of fluid (Stewart et al., 1997). The majority of bacteria found in the rumen are obligate anaerobes, meaning

that they grow strictly in oxygen-free conditions. However, some facultative anaerobes scavenge oxygen that enters the rumen with ingested feed (Hobson and Stewart, 1997). The diverse enzymatic function of bacteria contributes to the majority of metabolic activity found within the rumen. Some of the major functional classes of rumen bacteria include: fermenters of structural carbohydrate, nonstructural carbohydrate and organic acids, proteolytic species, lipolytic species, and β-hydrogenating species (Van Soest, 1994). While some rumen bacteria are highly specialized to require specific nutrients for growth, several species exhibit multiple functions and the ability to utilize a diverse set of nutrients. Rumen bacteria share a great degree of functional redundancy, so elimination of one species or group of species will likely have a great impact on rumen fermentation (Morgavi et al., 2012). In general, the rumen bacterial population is both cooperative and competitive, with many bacteria relying on the products of other to survive, and others using evolutionary niches to contend for resources.

Protozoa

Rumen ciliated protozoa are typically much larger than bacteria, and can vary widely in size from approximately 5 to 250 μm in length (Williams and Coleman, 1997). Originally described as “animalcules”, protozoa are eukaryotic with an outer membrane and an internal one-compartment digestive tract (Morgavi et al., 2013). Although scientists have been aware of the presence of protozoa in the rumen since the early history of rumen microbiology, there is still much to learn about their function within the rumen. Several studies used defaunation, removal of the protozoal population, to disclose that protozoa are not essential for rumen fermentation to occur (Akkada et al., 1968; Orpin, 1977b; Jouany et al., 1988). Rumen defaunation can drastically alter metabolism

in various ways. Studies have established that protozoal removal can decrease ammonia nitrogen and methanogenesis, while increasing feed conversion efficiency, especially in animals fed high forage diets (Kamra, 2005). However, protozoa may be beneficial in that they aid in nitrogen recycling within the rumen and can modulate rumen pH to protect animals fed high-starch diets from acidosis (Russell, 2002). The overwhelming majority of rumen protozoa are ciliated, although a few species of flagellates do exist (Hobson and Stewart, 1997). Ciliated protozoa are divided into two major classes: Holotrichs and the Entodiniomorphs. Holotrichs are characterized by cilia covering their entire body, while Entondinia contain cilia in discrete areas such as the mouth or posterior (Williams and Coleman, 1997). Both classes prey on rumen bacteria, and Entondiomorphs are known to cannibalize other protozoa (Hobson and Stewart, 1997). Protozoa grow at a slower rate than liquid phase retention time, but complete washout of protozoa is prevented by their adherence to the particle fraction of rumen digesta or to rumen epithelium.

Archaea

The contribution of archaea to rumen fermentation is relatively low. Archaea comprise approximately 3 to 4% of the total microbes in the rumen and constitute around 0.3 to 3.3% of the small-subunit rRNA in the rumen (Janssen and Kirs, 2008; Kong et al., 2013). Despite their low abundance, archaea are metabolically important in the rumen because of their role as producers of methane. Because they produce methane, archaea are a target for the reduction of greenhouse gas emissions from ruminants. Diversity of archaea is low compared with that of bacteria and protozoa. Eight methanogenic species from 5 genera have been cultured from the rumen (Kong et al., 2013), however, it is

likely that more species exist. Methanogens have a notorious reputation for being difficult to culture, and the existence of other rumen archaeal species has been suggested by molecular experiments (Janssen and Kirs, 2008; Krause et al., 2013). Of the primary methanogenic species found in the rumen, nearly two-thirds of the total archaea are within the Methanobrevibacter genus (Morgavi et al., 2012). Methanogens can be found free-floating in rumen fluid, attached to feed particulate matter or attached to the rumen epithelium. Additionally, they are close endo and ecto-symbionts with rumen protozoa which allows them to utilize the large amounts of hydrogen produced by protozoa as a substrate for methanogenesis (Janssen and Kirs, 2008). While archaea are principally known for their role in methane production it is increasingly likely that they may fill other niches in the rumen as well. Tajima et al. (2001b) used 16S rRNA sequencing to discover a novel group of archaea closely related to the non-methane producing *Thermoplasma* genus.

Fungi

The discovery of anaerobic fungi in the rumen came much later than the other clades in the rumen. While flagellates were observed in the rumen as early as 1910, all were originally thought to be protozoa. It was not until the 1970's when some flagellated zoospores were determined to be fungi (Bauchop, 1979). This discovery was confirmed by their growth into mycelium during culture and the presence of chitin in their cell wall, a characteristic only found in fungi (Orpin, 1975, 1977a). There have been 11 different species of rumen fungi identified, all of which are from the *Neocallimastix, Caecomyces, Orpinomyces, Ruminomyces,* or *Piromyces* genera (Kamra, 2005). Although the role of anaerobic fungi in the rumen is still not completely known, there is evidence to suggest

that they are important for fiber digestion. Scanning electron microscope images have shown that rumen fungi tend to attach themselves to the most lignified portion of plant tissues (Akin and Rigsby, 1987). Further evidence has demonstrated that fungi are able to penetrate the cuticle and cell wall of plant tissues and hydrolyze cellulose from within the plant (Varga and Kolver, 1997) The enzymatic profile of various rumen fungi indicate lignocellulase activity (Varga and Kolver, 1997). Studies have demonstrated that removal of fungi from rumen inoculum causes a significant reduction in *in vitro* gas production and degradation of fibrous feedstuffs (Kamra, 2005). Fungal growth in the rumen is inhibited by high-concentrate diets. This inhibition is likely due to a low rumen pH that is associated with concentrate feeding and inhibits growth of fungal zoospores in the rumen (Orpin, 1994).

Use of Molecular Techniques to Measure Rumen Microbial Populations

Historically, researchers relied on anaerobic culture-based techniques to study rumen microorganisms. Robert Hungate, considered the "Father of Rumen Microbiology", used his roll tube technique to discover a wide range of rumen bacteria and protozoa (Hungate, 1969). Research based on his techniques provided much of the current knowledge about rumen microbes. Growing pure and mixed cultures of microorganisms provided information regarding substrates, products and interactions between microorganisms in the rumen and provided a foundation to understand rumen fermentation. However, culture techniques rely on providing appropriate substrates and growing conditions, which becomes challenging when studying microorganisms from an environment as diverse and complex as the rumen. The dawn of molecular biology and the development of nucleotide-based sequencing technologies provided the ability for

culture-independent characterization of the rumen microbiome. Studies using microbial genomics have revealed that as few as 5 to 10% of bacterial phylotypes have been identified using standard culture-based or microscopic techniques (Firkins et al., 2008). Microbial genomics also divulged that the extent of certain known bacterial species may not be as high as previously accepted. For example, the *Ruminococcus* genus, traditionally thought to be one of the most important groups of cellulolytic bacteria, appeared a concentration no greater than 2% of total fiber-adherent rumen bacteria when quantified using DNA probes (Krause et al., 1999). Resistance to culture of many microbial species, combined with decreasing costs and increasing speed of DNA sequencing, has created a greater interest in using molecular techniques to examine the rumen ecosystem.

Oligonucleotide Primer Techniques

Since the 1980's, the 16S ribosomal RNA (rRNA) gene has been used to establish phylogenetic differences between bacteria. The 16S rRNA gene was first selected as a phylogenetic marker by Carl Woese because of its characteristic ability to be directly sequenced using a reverse transcriptase. It was identified as an ideal candidate gene for phylogenetic characterization because it contains both highly conserved and hypervariable regions, making evolutionary relationships easy to distinguish (Woese, 1987). Because of these characteristics, the gene was instrumental in illustrating divergence between eubacteria and archaea, and has since been used as the primary target for microbial ecological studies (Woese et al., 1990; Krause et al., 2013). Initial studies using the 16S gene involved the creation of synthetic oligonucleotide probes which targeted specific bacterial species of interest (Stahl et al., 1988). Short DNA sequences of

13 to 26 base pairs (bp) in length can be labeled with radioisotopes or flourescent dyes and allowed to hybridize to complementary sequences in microbial DNA (Stahl et al., 1988; Amann et al., 1990). The amount of hybridized primer can be enumerated to determine relative concentration of each species of interest. This technique has been used to describe competitive relationships between cellulose-degrading rumen bacteria (Odenyo et al., 1994a,b; Weimer et al., 1999) and has helped characterize the relationship between ruminal ciliated protozoa and endosymbiotic methanogens (Finlay et al., 1994). Oligonucleotide probes have discovered similarities in populations of *F. succinongenes* and archaea between natural rumens and continuous culture rumen fermenters (Ziemer et al., 2000).

Oligonucleotides probes can also be used in a technique called Fluorescence *in situ* hybridization (FISH), where fluorescent DNA or RNA molecules are added to cultures of fixed and permeabilized microbial cells. Fluorescent probes enter cells, bind to the 16S gene and are viewed with a fluorescence microscope (Moter and Göbel, 2000). Because this technique allows intact microbial cells to be imaged, it is particularly useful for determining the spatial relationships between organisms. Fluorescence *in situ* hybridization has been used to determine adherence sites of fibrolytic bacteria within fibrous plant material (Shinkai and Kobayashi, 2007), quantify methanogenic archaea within the rumen of sheep (Yanagita et al., 2000), observe the relationship between protozoa and their bacterial and archaeal endosymbionts (Lloyd et al., 1996) and quantify mucosa-adhered microbes in young lambs and calves (Collado and Sanz, 2007). Unfortunately, this technique relies on growing microbes of interest, so it fails to avoid the problem of culture bias.

Polymerase Chain Reaction

Development of the polymerase chain reaction (PCR) was a major step in all sectors of molecular biology, including rumen microbial ecology. During the PCR procedure, primer-targeted DNA strands are cloned through thermocycling with a heat-resistant DNA polymerase and the addition of deoxynucleotide phosphate molecules (dNTPs) (Innis et al., 1988). This process creates millions of copies of a particular DNA segment or gene of interest which is particularly useful when a low quantity of DNA is acquired from a sample. Clones generated from PCR amplification have a number of downstream applications including DNA fingerprinting and genomic sequencing. Microbial ecological studies use PCR to amplify regions of the 16S gene of bacteria and archaea and the 18S rRNA gene of eukaryotes such as fungi to generate gene 'libraries' which can be fully or partially sequenced (Fouts et al., 2012). This technique has been used to identify the dominant species of *Prevotella, Bacteriodes* and methanotrophic archaea in the rumen (Wood et al., 1998; Mitsumori et al., 2002). DNA may also be quantified simultaneously to amplification using a technique called quantitative real-time (qPCR). In this process, molecular beacons are used to monitor the progress of the PCR reaction in real-time, allowing for more accurate determination of absolute and relative quantities of nucleic acids within a sample (Filion, 2012). Tajima et al. (2001) used qPCR to demonstrate dramatic decreases in *F. succinogenes* and *R. flavefaciens* along with substantial increases in *Prevotella spp.* when Holstein cows were switched from a high forage to a high concentrate diet. Ozutsumi et al. (2006) also used this approach to determine the effects of ruminal defaunation on the abundance of *F. succinongenes, R. albus, R. flavefaciens, P. ruminocola, P. albensis* and *P. bryantii*. A modification of

qPCR involves co-amplifying target DNA with known amounts of competitor DNA possessing a similar nucleotide sequence to the target. This method, quantitative competitive (QC-PCR), allows for more precise determination of an absolute number of DNA molecules within a sample (Sirohi et al., 2012). QC-PCR has been used to determine the levels of cellulolytic bacteria (Koike and Kobayashi, 2001), anaerobic fungi (Sekhavati et al., 2009) and proteolytic bacteria (Reilly et al., 2002).

DNA Fingerprinting

Differences in microbial community structure can be examined using DNA fingerprinting techniques, which are typically less costly and time consuming than genomic sequencing, but lack the ability to assess species richness or diversity (Bent et al., 2007). Community fingerprinting involves separation of DNA fragments based on their length or chemical properties using gel electrophoresis, which creates a unique banding pattern based on the microbial groups present, providing a crude illustration to compare and contrast microbial communities (Ramette, 2009). Fingerprinting techniques include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal-restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), and automated ribosomal intergenic spacer analysis (ARISA). DGGE separates double-stranded (ds) DNA fragments based on their electrophoretic mobility in a gel containing a linear gradient of urea and formamide (Muyzer et al., 1993). DGGE has been used to compare microbial communities fed varying levels of grain (Kocherginskaya et al., 2001) and differentiate microbial communities between animals with differing residual feed intake (RFI) (Guan et al., 2008; Hernandez-Sanabria et al., 2010, 2012). It has also been utilized to describe

the unique community attached to the rumen epithelium (Sadet et al., 2007) and study diversity of methanogens in diets containing condensed tannins (Mohammed et al., 2011) or chloroform (Knight et al., 2011).

Terminal-restriction fragment length polymorphism analysis begins by digesting fluorescently-labeled PCR amplicons with restriction enzymes. DNA fragments are separated based on the position of the restriction site using gel electrophoresis (Marsh, 1999). A fluorescence detector generates a series of peaks corresponding to size and relative abundance of these fragments (Sirohi et al., 2012). T-RFLP has been used to model changes in the microbiome during subacute ruminal acidosis (SARA) in dairy cattle (Khafipour et al., 2009) and correlate bacterial concentrations with VFA levels in transition dairy cows (Wang et al., 2012). Amplified ribosomal DNA restriction analysis is an extension of T-RFLP which uses restriction enzymes specific to the 16S rRNA gene (Vaneechoutte et al., 1992). ARDRA has been used to investigate the effects of the bacteriocin Bovicin HC5 in ruminal mixed culture (Lima et al., 2009), plant extracts in continuous culture fermenters (Ferme et al., 2004) and survey diversity of rumen protozoa in sheep (Regensbogenova et al., 2004). Typically, ARDRA is preferred when a microbial community is dominated by a small number of species (Sirohi et al., 2012). Automated ribosomal intergenic spacer analysis uses a similar method to T-RLFP, but detects the intergenic region between ribosomal genes rather than the genes themselves (Fisher and Triplett, 1999). ARISA been used for a variety of applications including describing relationships between the host and microbiome (Weimer et al., 2010), examining rumen community dynamics across the daily feeding cycle of dairy cows (Welkie et al., 2010) and determining the diversity of ruminal fungi (Denman et al., 2008;

Edwards et al., 2008). ARISA was found to be more accurate in detecting changes to rumen bacteria than DGGE and T-RFLP, but has a more limited database of fingerprint profiles than T-RFLP (Saro et al., 2014; Danovaro et al., 2006).

DNA Sequencing

While community fingerprinting techniques are useful for providing imprecise comparisons between microbial communities, these techniques are limited in their ability to quantify microbial diversity or identify microbial species present because of their non-targeted approach. Furthermore, probe- and primer-based techniques are limited by the availability of known sequences for probe or primer generation. Presently, the majority of the primers available are for species that have been cultured, limiting the ability for enumeration of uncultivable species (Morgavi et al., 2013). A more specific approach to estimating diversity of microbial communities involves direct sequencing of phylogenetic marker genes, allowing the full complement of microbes from a community to be characterized into taxonomic groups. The advent of high-throughput sequencing techniques, often referred to as *next-generation* sequencing, has greatly improved speed and quantity of genomic data that can be generated from a biological sample. Prior to the development of high-throughput techniques, Sanger shotgun sequencing was the gold-standard for genomic sequencing (Sanger and Coulson, 1975). However, Sanger sequencing is time consuming, expensive, suffers from cloning bias due to the necessary growth of plasmid vectors and has difficulty sequencing regions with long repeated DNA regions (Wooley et al., 2010). In contrast, high-throughput sequencing has the ability to rapidly generate an enormous amount of genomic data at a much lower cost than Sanger sequencing.

Several high-throughput sequencing methods have been developed, including the Roche 454 GS FLZ, the Illumina HiSeq and MiSeq systems, the Ion Torrent system, and the Applied Biosystems SOLiD platform (van Dijk et al., 2014). Although each system applies a different approach to DNA fragment sequencing, all operate on the principle of developing clusters of PCR amplicons derived from a single nucleic acid molecule (Wooley et al., 2010). Millions of amplicon clusters may be simultaneously generated during amplification, helping to improve resolution of the final sequence. After cluster generation, sequencing is executed using one of two methods: sequencing by synthesis or by ligation (Hodkinson and Grice, 2015). The Roche 454, Illumina and Ion Torrent systems use the sequencing-by-synthesis method that involves the addition of a DNA polymerase and labeled nucleotides adenine (A), thymine (T), guanine (G) and cytosine (C). When nucleotides are polymerized to DNA amplicons, a chemical or fluorescent signal is released and simultaneously detected by a high-resolution camera (Thomas et al., 2012). This process involves all clusters simultaneously, allowing millions of different DNA fragments to be sequenced in parallel. In the sequencing-by-ligation method, which is performed by the SOLiD platform, a ligase and several fluorescently labeled DNA octamers (eight-base pair long sequences) are added to a sample. Fluorescent signals are transmitted as DNA octomers are ligated to one another after they have been polymerized to their complementary sequences in the sample DNA (Pandey et al., 2008). While a more comprehensive description of the individual sequencing technologies is beyond the scope of this paper, several reviews are available that describe each of them in greater detail (Metzker, 2010; Mardis, 2008). All high-throughput sequencing methods computationally convert detected signals into a string of A's, T's,

G's and C's, corresponding to the sequence of the four possible nucleotide bases. These sequences must then undergo bioinformatic analysis to filter the sequences and determine the species present within each sample. At the present time, the ROCHE 454 GS FLZ and Illumina HiSeq/MiSeq platforms are most commonly used for rumen microbiome studies (McCann et al., 2014a). In the future, there may be greater development and use of single-molecule sequencing technologies that sequence DNA without the use of PCR amplification, which would lower costs and eliminate amplification bias.

16S Amplicon Sequencing Analysis

16S amplicon sequencing is a revolutionary approach that uses high-throughput genomic sequencing to describe the ecology of complex microbial communities. This technique uses generalized primers to sequence a specific region of the 16S gene of all microbial species present, and uses computational tools to determine their phylogeny. The steps in 16S amplicon sequence analysis include sampling, extraction, library preparation, sequencing, assembly, annotation and statistical analysis (Figure 1.2). During sampling, consideration must be given to the technique, time relative to feeding and region of the rumen from where samples were collected. Li et al. (2009b) compared microbial community profiles obtained from five sampling locations within the rumen at three different sampling times. Their experiment observed differences ($P < 0.05$) in microbial communities based on time of sampling, not based on location within the rumen. Lodge-Ivey et al. (2009) compared bacterial diversity measurements between cows sampled either with an oral lavage or through a rumen cannula and found that both methods produced similar microbial profiles. However, the findings from these studies contrast with others that have detected discrepancies in fermentation parameters between

the two sampling methods, particularly pH and volatile fatty acid levels (Geishauser and Gitzel, 1996; Shen et al., 2012).

After sample collection, DNA must be extracted from microbial cells. Several extraction methods exist, and the method used can influence the apparent microbial community based on the lysis and clarification steps used. Henderson et al. (2013) compared fifteen different DNA extraction methods for sheep and cattle rumen samples and found variation in communities extracted using different techniques. Therefore, it is strongly advised to resist comparing studies with different extraction methods. Ideally, an extraction method for all ruminant studies should be standardized. Extracted DNA is used to generate DNA libraries by fragmenting or amplifying a region of the target gene, and adding adapter and barcode sequences to the DNA fragments (Wooley et al., 2010). The targeted gene region is nearly always one of the hypervariable regions within the small subunit rRNA gene (McCann et al., 2014a). Pitta et al. (2014) compared the V1-V3, V4-V5, and V6-V8 hypervariable regions of the 16S rRNA gene of bacteria collected from *Bos indicus* cattle. Different hypervariable regions resulted in greatly divergent microbial profiles and the V1-V3 region provided the strongest representation of species richness and diversity in the rumen. For many high-throughput sequencing techniques, adapter sequences and barcodes are added during the library preparation step. Adapters are short nucleotide sequences which bind to the complimentary oligonucleotides of a sequencing platform to allow for cluster generation (Mardis, 2008). Barcodes are unique DNA sequences that may be added with the adapter, allowing evaluation of many different communities in a single sequencing run (McCann et al., 2014a). This process, known as

multiplexing, uses different barcode sequences for each community to distinguish between then during analysis (Parameswaran et al., 2007).

Once the library is prepared, fragments undergo high-throughput sequencing to generate the order of base pairs in the sample. A number of computational steps must be performed to analyze the DNA sequences. Initially, DNA must be filtered to remove short, ambiguous, low quality or chimeric sequences (Schloss et al., 2011). This prevents many possible downstream errors that could occur during sequence assembly and analysis. An assembly algorithm is used to take short sequence reads produced from sequencing and align them to form longer contiguous sequences called *contigs*. (Miller et al., 2010). Assembly can be performed using a reference database or *de novo*, depending upon available data on the microbiome and the goals of the experiment. Reference-based assembly uses a previously established dataset of similar microbiomes to identify the organisms present (Thomas et al., 2012). If no reference database is present, or if the experiment aims to investigate groups in a microbiome that are poorly described, *de novo* assembly must be performed. This process is more computationally intense, but is able to assemble metagenomes without previous knowledge of the microbial community (Miller et al., 2010). It is important to realize that with either method, full assembly of a gene region is frequently not possible, especially in diverse microbial communities, so analysis is typically done on incomplete sequences (Wooley et al., 2010). After metagenome assembly, sequence reads are clustered into operational taxonomic units (OTUs) in a process called binning (Thomas et al., 2012). Typically, an OTU with 97% similarity to a reference sequence is considered a proxy for species (McCann et al., 2014a). These OTUs are then compared against databases such as Greengenes, SILVA and Ribosomal

Database Project (RDP) to determine species richness and diversity (Thomas et al., 2012). Prediction of the microbial function can be made based on the OTU's present using computer software such as PICRUSt (Langille et al., 2013).

Applications of High Throughput Sequencing in Ruminant Studies

High-throughput sequencing of rumen microbes has been used in a wide variety of studies. Several have focused on correlating certain microbiome species with economically important traits such as milk production and feed efficiency. McCann et al. (2014b) used Roche 454 GS FLX pyrosequencing to compare the microbiome of high-RFI and low-RFI Brahman bulls. They found that *Prevotella* species were more abundant in high RFI bulls and an unidentified *Bacteriodales* family was found at a high concentration in bulls with low RFI. High-throughput 16S amplicon sequencing has revealed a strong correlation between high *Firmicutes*:*Bacteroidetes* ratio and greater milk fat production of lactating Holstein cows (Jami et al., 2014). High-throughput sequencing has also described microbial taxa responsible for development of common metabolic disorders that affect livestock. For example, SARA has been found to be associated with a decrease in overall bacterial diversity and an increase in *S. Bovis, Lactobacillus spp.* and *Succiniclasticum spp.* (Steele et al., 2011; Petri et al., 2013; Huo et al., 2014). Other disciplines, especially those in the biofuel industry have used sequence analysis of the rumen microbiome to discover potentially novel biocatalysts (Steele et al., 2009). Hess et al. (2011) discovered 27,755 putative carbohydrate active-enzymes in the rumen of cows fed switchgrass, demonstrating the rumen's immense potential as a biocatalyst source.

In the future, as the quantity of data generated from high throughput sequencing increases and the cost decreases its use for characterization of the rumen microbial population will become more widespread. As a greater number of rumen microbial genomes are sequenced, a more complete database of organisms will be generated, allowing for better characterization of rumen microbiomes. Currently, the Hungate1000 project attempting to fully catalogue the genomes of 1000 different rumen organisms with the intention of improving the accuracy of rumen ecological data. At present time, the project has successfully sequenced the genomes of 271 rumen microorganisms with another 234 genome sequences currently in progress (http://www.hungate1000.org.nz/). In the future, research will focus more on the functional capabilities of the rumen microbiome by looking at global gene (metatranscriptomics) and protein (metaproteomics) expression, rather than simply phylogeny (McAllister et al., 2015). These technologies will allow greater insight into the metabolic capabilities of the microbial community at a molecular and enzymatic level, which will allow nutritionists and rumen microbiologists to better manipulate the microbial population through dietary and non-dietary factors.

MANIPULATION OF RUMEN MICROBES

Goals of Rumen Fermentation

The focus of ruminant livestock production is regulating rumen fermentation to promote the most efficient conversion of feed energy into meat, milk or wool. To maximize production output, an ideal rumen fermentation must promote rapid rates of fiber degradation, efficient production of microbial protein and an optimal ratio of VFA (Russell, 2002). Efficient production also strives to minimize methane and ammonia

which represent wasted nutrients to the animal and are associated with environmental pollution. Lactic acidosis must be avoided to prevent detrimental effects on the microbial community, animal health and fiber degradation (Plaizier et al., 2008). Initial attempts to achieve these goals were done through dietary manipulations, but with greater knowledge regarding rumen microbes and their ecosystem, more targeted antibiotic approaches have been used (Bergen and Bates, 1983).

Methane represents an energetic loss to the ruminant animal and a contribution to greenhouse gas emissions. It is estimated that rumen methanogenesis results in a six to ten percent loss of gross energy (Cottle et al., 2011). Moreover, ruminants have been implicated as the source for about 15% of total atmospheric methane emissions (McAllister et al., 1996). Therefore, reducing enteric methane is a concern of most ruminant livestock producers. Methanogenesis is a normal process in the rumen that occurs as a disposal mechanism for hydrogen. During fermentation of sugars via the Embden-Meyerhof-Parnas pathway, NAD^+ is reduced to NADH, which must be oxidized to regenerate NAD^+ for the pathway to continue (McAllister and Newbold, 2008). Due to the anaerobic environment of the rumen, O_2 cannot be used as a final electron acceptor, so many of the free electrons are used by methanogens to reduce CO_2, forming methane (CH_4) (McAllister et al., 1996). Strategies for reducing the production of methane typically involve altering fermentation to promote the use of alternative pathways for this hydrogen disposal (Figure 1.3). A common alternative is to shift fermentation in favor of propionate production, which unlike acetate is a consumer of free hydrogen (Martin et al., 2010). Other hydrogen disposal mechanisms include biohydrogenation of fatty acids,

nitrate reduction, sulfate reduction and reductive acetogenesis (McAllister and Newbold, 2008).

Ammonia is necessary for growth of most rumen microbes and for synthesis of microbial protein that can be utilized by the animal. However, excess ammonia production due to high levels of amino acid deamination represents a waste of dietary nitrogen supplied to the animal. An overabundance of absorbed ammonia may also contribute to a waste of metabolizable energy, due to the energetic cost of detoxifying plasma ammonia by ureagenesis in the liver (Canfield et al., 1990; Wathes et al., 2007). While balancing available carbohydrate and protein can greatly reduce the amount of unutilized nitrogen in the rumen, ammonia accumulation can still occur (Bach et al., 2005). A small group of Gram-positive bacteria, often called hyper-ammonia producing bacteria (HAB), are responsible for contributing to the majority of amino acid deamination and ammonia production in the rumen (Flythe, 2009). Species of HAB include *Peptostreptococcus anaerobius, Clostridium stricklandii, Clostridium aminophilum, Fusobacterium necrophorum* and other unidentified species of *Bacteriodaceae* and *Eubacterium* (Attwood et al., 1998; Rychlik and Russell, 2000). Collectively, these bacteria produce up to 20 times more ammonia than other amino acid degrading bacteria (Paster et al., 1993). Selective inhibition of these microbes can greatly reduce protein wasting and improve efficiency of nitrogen utilization by the animal (Chen and Russell, 1989; Flythe et al., 2013).

When excess fermentable carbohydrates are provided to the diet of a ruminant, ruminal acidosis may occur. Acidosis is characterized by a prolonged period of low rumen pH, usually accompanied by an accumulation of lactate. When carbohydrates are

very rapidly fermented, high levels of lactate and VFA are formed that may not be adequately buffered by saliva, causing rumen pH to drop (Plaizier et al., 2008). Persistently low rumen pH promotes the growth of lactic acid bacteria, such as *Streptococcus bovis*, that thrive in the acidic environment (Khafipour et al., 2009). These organisms continue to reduce rumen pH, perpetuating an acidotic condition. Rumen acidosis has a multitude of negative effects in the rumen and to the host animal. Fiber-degradation is greatly inhibited because of the high acid-sensitivity to most fibrolytic bacteria (Russell and Wilson, 1996). Low rumen pH also has detrimental effects on rumen papillae, causing inhibition of fermentation product absorption (Steele et al., 2009b). Compromised epithelial barrier function also allows endotoxins from the rumen to enter the animal's bloodstream, causing an inflammatory response (Li et al., 2012). Ultimately, these conditions can result in inhibited milk and milk fat production, laminitis, liver abscesses and metabolic syndrome (Plaizier et al., 2008). Treating acidosis is difficult because of the dramatic shift in microbial population, however, the addition of feed additives to inhibit growth of lactic acid bacteria or promote growth of fibrolytic bacteria can mitigate some of the effects of the disease (Newbold and Wallace, 1988; Beauchemin et al., 2003).

Selective Promotion of Rumen Microbes

A renewed focus on the relationship between the rumen microbiome and ruminant production have increased interest in use of probiotics and prebiotics in ruminant diets. Probiotics contain live cultures of metabolically important microbes which are added to the diet with the hopes of improving rumen fermentation. Yeasts, in particular, have received significant attention as a potential feed additive for ruminants. *Saccharomyces*

cerevisiae has been demonstrated to increase milk production, dry matter intake (DMI), DM degradation, and NDF digestibility in some studies (Williams et al., 1991; Plata et al., 1994; Guedes et al., 2008; Yalcin et al., 2011), but results, particularly with respect to fiber digestion, have been inconsistent (Yoon and Stern, 1996; Doreau and Jouany, 1998). Desnoyers et al. (2009) performed a meta-analysis on 157 different experiments where *S. cerevisiae* was supplemented to ruminants and found that its dietary inclusion decreased rumen lactate concentration, increased DMI, total tract OM digestibility, rumen pH and total VFA, and tended to increase milk production and milk fat %. Furthermore, the experiment found that the effect on rumen pH was more pronounced with a high percentage of concentrate in the diet (Desnoyers et al., 2009). This observation is consistent with several studies that established the ability of *S. cerevisiae* supplementation to alleviate subacute ruminal acidosis (AlZahal et al., 2014; Vyas et al., 2014). Microbial culturing (Callaway and Martin, 1997) and metagenomic sequencing (Pinloche et al., 2013) revealed that supplementation with *S. cerevisiae* increases the proportion of fiber-degrading and lactate-utilizing bacteria in the rumen. The saccharide-degrading mold *Aspergillus oryzae* is another fungal species that has been used as a probiotic with the ability to increase hemicellulose (Wiedmeier et al., 1987) and total tract fiber digestion (Gomez-Alarcon et al., 1990) as well as increase numbers of cellulolytic (Wiedmeier et al., 1987; Yoon and Stern, 1996) and proteolytic bacteria (Yoon and Stern, 1996).

Several bacterial species and strains have been studied as potential probiotics for ruminants. Lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium* and *Enterococcus* species have been included in the diets of cattle and may help prevent ruminal acidosis

through promoting the growth of microbes adapted to the presence of lactate (Elghandour et al., 2015). Lactate-utilizing bacteria, such as *Megasphaera elsendii* or *Propinibacterium spp*., may be supplemented to prevent high ruminal lactate levels (Chaucheyras-Durand and Durand, 2010). *Ruminococcus flavefaciens* NJ has been shown to increase cellulolytic bacterial populations and improve DM digestibility of timothy hay fed to dairy calves (Chiquette et al., 2007). Supplementation of *Prevotella bryantii* 25A during the periparturient period of dairy cows precipitated an increase in acetate propionate, and butyrate concentrations and resulted in greater milk fat compared with controls (Chiquette et al., 2008). Utilization of metagenomic sequencing has greatly advanced the ability to determine the impact of specific probiotics on the rumen ecosystem. For example, Præsteng et al. (2013) found that the administration of *R. flavefaciens* induced an increase in *Prevotella* and a decrease in uncharacterized *Bacterodetes*. Further, *In silico* identification of potentially novel microbial species and enzymes has created an opportunity to discover potential probiotic organisms (Gilbert et al., 2015). Some attempts to produce genetically modified probiotics for use in ruminant feeds have been made. Liu et al. (2005) successfully developed a recombinant strain of *Lactobacillus reuteri* that expressed additional xylanase, β-glucanase, and cellulase genes. However, applicability of genetically modified rumen bacteria has been debated because of limited available niches in the rumen, the difficult transcriptional regulation process of bacteria and concerns regarding use of genetically modified organisms in food production (Russell, 2002).

Selective Inhibition of Rumen Microbes

Many compounds of plant origin have antimicrobial action in the rumen. While the specific mechanism of action of many of these compounds is unknown, it is believed that nearly all of them exert their effects by interacting with processes associated with the bacterial cell membrane (Benchaar et al., 2008). The cell membrane of microorganisms is important for maintaining the intracellular environment of microbes and for production of cellular energy (Russell and Strobel, 1989). Gram-negative bacteria generally experience less growth inhibition from compounds that impair cell membrane function because their outer cell membrane is more typically more impermeable to large molecules than that of Gram-positive bacteria and other microorganisms (Russell and Strobel, 1989).

Ionophores

In the United States, ionophores are frequently used in cattle diets as antimicrobial feed additives. Monensin is the most commonly used ionophore in ruminant diets, but others including lasalocid, tetronasim, lysocellin, narasin and laidlomycin have been used commercially or evaluated for potential use (Russell and Strobel, 1989). These compounds are lipophilic, allowing them to dissociate into the lipid membranes of bacterial cells (Schelling, 1983). They interact with metal cations, transporting them across the lipid bilayer (Ovchinnikov, 1979). All microorganisms rely on maintaining an ionic gradient across their cell membrane for survival. Microbes use ATPases or electron transport systems found in their cell membrane to translocate protons across the cell membrane and establish a proton motive force (Russell and Strobel, 1989). This proton motive force is used to drive ATP synthases for cellular energy production. When ionophores enter the bilayer, they transport sodium ions or

protons into the cell which disrupts the proton gradient (McGuffey et al., 2001). To compensate for loss of proton motive force and decreased intracellular pH, microbes increase transmembrane ATPase activity, depleting the cellular levels of ATP and inhibiting microbial growth (Russell, 2002).

In general, the addition of ionophores to diets favors the growth of Gram-negative bacteria relative to Gram-positive bacteria, however, this is not always the case. Some Gram-negative bacteria are more sensitive to ionophores, possibly due to bacterial cell wall structure or effects on glucose transport mechanisms (McGuffey et al., 2001). Chen and Wolin (1979) also substantiated that certain Gram-positive bacteria have low sensitivities to ionophores. Rumen fungi are also sensitive to ionophores in pure culture and *in vivo* (Elliott et al., 1987). Protozoa appear to be sensitive to ionophores, but the degree of sensitivity depends greatly on species and diet composition (McGuffey et al., 2001). Some microbes can adapt to the presence of ionophores by developing resistance mechanisms. For example, *P. bryantii* B14 is documented to require 16-fold more monensin to inhibit cell growth, compared with unadapted strains of the bacteria (Callaway and Russell, 1999).

Ionophores have consistently elicited increases in the molar proportion of propionate with simultaneous decreases in acetate and butyrate (Bergen and Bates, 1983), which is partially explained by replacement of Gram-negative bacteria with Gram-positive bacteria (McGuffey et al., 2001). An increase in propionate relative to acetate is typically associated with a decrease in methane production because of the consumption of free hydrogen during propionate synthesis (McAllister et al., 1996). The decrease in methane may also be caused by inhibition of protozoa, which constitute the largest

microbial producers of hydrogen in the rumen. While ionophores consistently decrease methane production, they have no toxic effect on methanogens themselves (Russell and Strobel, 1989). Many studies have demonstrated that ionophores decrease rumen ammonia and microbial protein production, primarily due to inhibited amino acid deamination (Chen and Russell, 1991; McGuffey et al., 2001). The inhibition of amino acid deamination that occurs when ionophores are fed may cause a greater percentage of metabolizable protein to come from dietary true protein, rather than microbial protein (Tedeschi et al., 2003).

Plant Secondary Metabolites

Public concern regarding the use of antibiotic growth promotants has grown in recent years. Using antibiotics at sub-therapeutic levels in livestock has been criticized as a possible source of multiple drug resistant bacteria (Benchaar et al., 2011). As of 2003, the European Union prohibited in-feed antibiotics, including ionophores, for use in livestock production (García-González et al., 2008). These concerns led to investigation for the use of natural plant compounds as alternative modifiers of rumen fermentation. Many plants produce antimicrobial compounds which are used for diverse ecological or defensive functions (Harborne, 1999). A multitude of these compounds have been studied for their effects on rumen fermentation and microbial growth.

Saponins are amphipathic steroid or triterpenoid glycosides that are found in numerous plant compounds (Francis et al., 2002). Steroidal saponins from *Yucca schidigera* extract have consistently inhibited growth of ruminal protozoa *in vitro* (Wallace et al., 1994; Wang et al., 1998; Narvaez et al., 2013), but *in vivo* studies have been more variable (Hristov et al.; Wallace et al., 1994; Eryavuz and Dehority, 2004).

Other studies assert that saponins can reduce rumen methane production because of a reduction in protozoal numbers (Pen et al., 2006; Holtshausen et al., 2009). Several compounds besides saponins have been studied for their potential antimicrobial properties. Cinnamaldehyde (cinnamon oil), eugenol (clove buds), capsaicin (spicy peppers), garlic oil, benzyl salicylate and anise oil precipitated increases in propionate production and decreases in acetate and/or methane production in rumen batch culture (Busquet et al., 2006; Calsamiglia et al., 2007). Furthermore, capsicum, carvacrol (thyme), carvone (caraway and dill), cinnamaldehyde, cinnamon oil, clove bud oil, eugenol, fenugreek (herb prominent in South Asia), and oregano oil reduced rumen ammonia-N concentration *in vitro* (Busquet et al., 2006). While these compounds have shown promise as modifiers of rumen fermentation, their mechanisms of action are not well-known and must be further evaluated.

Hops

Hops have promise as a potential anti-microbial to modulate rumen fermentation. Hops (*Humulus lupulus)* were used for centuries in the brewing industry as a preservative and flavor enhancer. The hop plant likely existed in ancient Egypt and Israel, however, its first recorded use was as a decorative plant in medieval Europe in AD 736 (Verzele, 1986). Growing hops was popularized in Germany in 1079, but they were not used as a beer additive until the 12th century (Verzele, 1986; Haas and Barsoumian, 1994). Brewers discovered that adding hops during brewing had antiseptic effects and prevented spoilage of beer (Van Cleemput et al., 2009). Hayduck (1888) was the first to discover the basis of the antimicrobial activities of hops. He extracted resin from hop cones and used lead acetate to precipitate and isolate two resin fractions. These two fractions, known as

humulones (α-acids) and lupulones (β-acids), were found to be responsible for conferring most of the bacteriostatic properties of hop cones (Hayduck, 1888).

Evidence has revealed that α- and β-acids can selectively inhibit growth of Gram-positive bacteria and fungi by disrupting the cellular membrane in a similar manner as ionophores (Haas and Barsoumian, 1994). *Trans*-isohumulone can divert protons across the cellular membrane into the intracellular fluid, disrupting the proton-motive force needed to drive ATP synthase (Simpson, 1993a). Other research suggests that *trans*-isohumulone is a mobile ion carrier that can exchange protons for divalent cations such as Mn^{2+}, leading to a reduction in intracellular pH which inhibits nutrient transport (Simpson, 1993b). However, recent potentiometric studies could not confirm the presence of H^{+}/Mn^{2+} exchange from the addition of isohumulone, but instead suggested a manganese-dependent enhancement of transmembrane charge permeation (Behr and Vogel, 2009). Further research using cyclic voltammetry detected that isohumolone and other hop constituents may affect oxidation/reduction status of intracellular manganese, leading to oxidative stress and cellular damage (Behr and Vogel, 2010; Schurr et al., 2015). In addition to their action in the cell membrane, research indicated that α- and β-acids can also play a role in inhibiting DNA, RNA and protein synthesis (Teuber and Schmalreck, 1973). Simpson (1993a) showed that *trans*-isohumulone decreased levels of leucine in the cell by reducing its uptake and increasing its extracellular release, which may partially explain the role of hop acids in reducing bacterial protein synthesis.

Bacteria have exhibited resistance mechanisms against hop acids, similar to ionophores and other membrane-active compounds. *Lactobacillus brevis* is a common beer-spoilage bacteria, that has developed multiple resistance mechanisms including

development of a multidrug transporter protein (Sakamoto et al., 2001), increased cellular H^+-ATPase activity (Sakamoto et al., 2002) and an intracellular acid defense system (Schurr et al., 2013). Proteomics has further advanced the understanding that *L. brevis* expresses a stress-related response and shifts its metabolism to save energy when encountered with hops (Behr et al., 2007). Hop resistance is largely dependent on pH because the ATPase found in the adapted organisms has its greatest activity at a pH of 5.6, but is markedly reduced at a pH of 6.6 (Sakamoto and Konings, 2003).

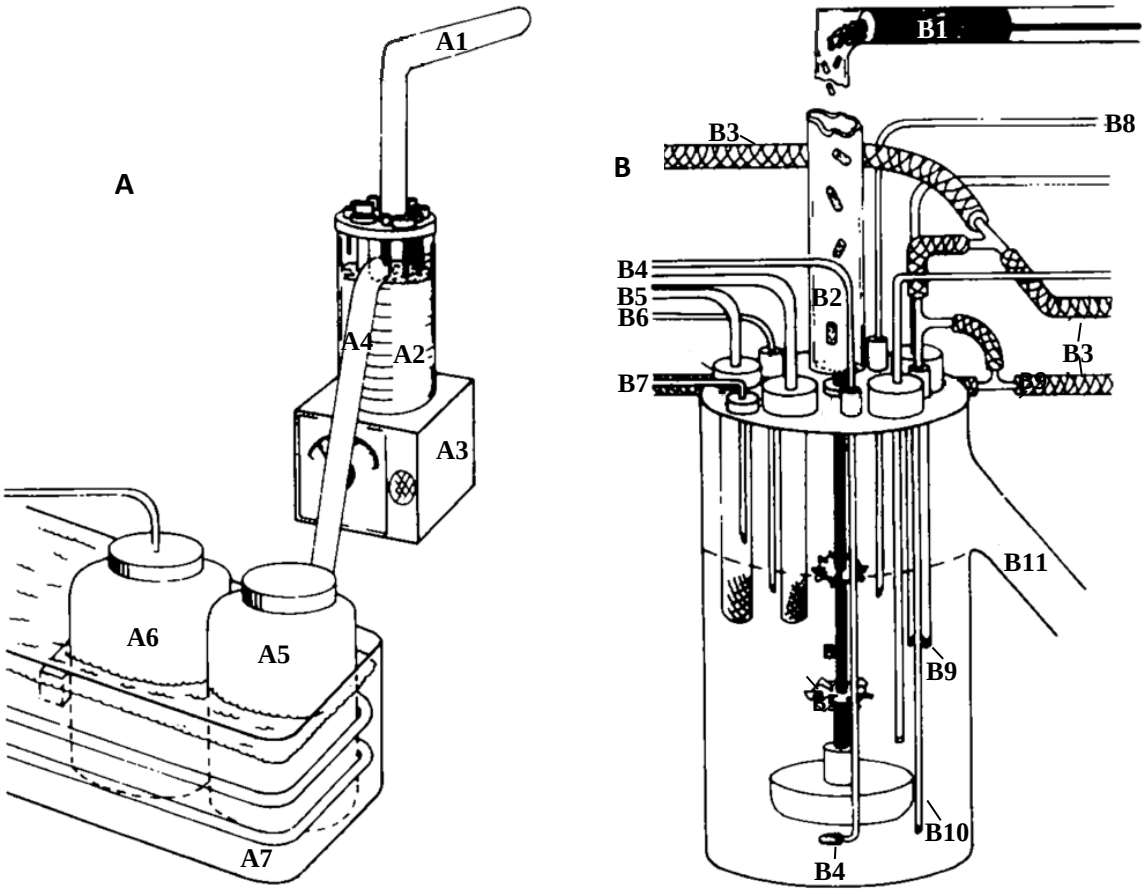
Hop α- and β acids have been investigated as a potential antibiotic feed additive for ruminant diets. Flythe (2009) noted that the addition of high-lupulone hops extract to cultures of mixed rumen microbes decreases the rate of ammonia production and inhibits growth of hyper-bacteria. Flythe and Aiken (2010) established that addition of hops extract to co-culture increased molar proportion of propionate and inhibited growth of lactate-producing bacterium *S. bovis*, suggesting that hop acids may be able to prevent rumen acidosis. *In vitro* batch culture studies provided supporting evidence that hop acids decrease methane and ammonia-nitrogen accumulation and favor propionate production (Wang et al., 2010). Microbial analyses using 16S rRNA quantification also revealed that hops extract reduced methanogenic archaea and *R. flavefaciens* (Narvaez et al. 2013). While *in vitro* analyses have been encouraging, *in vivo* analyses have been less favorable. Drouillard et al. (2009) found no effects ($P > 0.05$) on total bacteria, *S. bovis*, methanogens, VFA profiles or ruminal lactate when steers were fed 30 mg β-acids/kg of diet DM. Wang et al. (2010) found no impact on growth performance or carcass characteristics in steers fed hop pellets at a rate of 40 or 80 mg β-acids/kg of diet DM.

Lack of response seen in their study may be due to low inclusion levels of hop acids or adaption by rumen microbes.

SUMMARY

Achieving optimal rumen fermentation is a key component of maintaining healthy and productive ruminant livestock. However, the complex ecological relationship between rumen microorganisms makes it difficult to tailor fermentation to maximize the potential of the animal. Many compounds can be used to manipulate rumen fermentation by affecting the microbial population, but their mechanisms of action are not completely understood, and are complicated by microbial and environmental interactions. In addition, these compounds are often subject to microbial adaptation, reducing their effectiveness. The development of high-throughput molecular techniques has potential to greatly improve understanding of rumen microbe dynamics because of the extensive amount and high precision of data that is produced. In the future, these technologies will continue to improve in accuracy and decrease in cost, increasing their utility for ruminant nutrition studies. With these technological advancements, along with improvements in knowledge of feedstuffs, animal physiology and animal comfort, there is opportunity to advance the efficiency and productivity of ruminant livestock.

Figure 1.1. General schematic of dual flow continuous culture system. Modified from Hannah et al. (1986).



(A) General schematic of dual flow continuous culture fermenter system. **(A1)** Automated feed delivery system. **(A2)** Continuous culture fermentation vessel. **(A3)** Magnetic stir plate with temperature control. **(A4)** Solid effluent overflow tube. **(A5)** Solid effluent collection vessel. **(A6)** Liquid effluent collection vessel. **(A7)** Refrigerated water bath (2°C). **(B)** Fermentation vessel assembly. **(B1)** Feed delivery tube. **(B2)** Feed input port. **(B3)** Coaxial heat exchange apparatus. **(B4)** Nitrogen sparager. **(B5)** Liquid effluent filters. **(B6)** Hydrochloric acid input port. **(B7)** Buffer infusion port. **(B8)** Sodium hydroxide input port. **(B9)** pH electrode. **(B10)** Temperature probe. **(B11)** Solid effluent port.

Figure 1.2. Steps of 16S amplicon analysis of environmental samples.

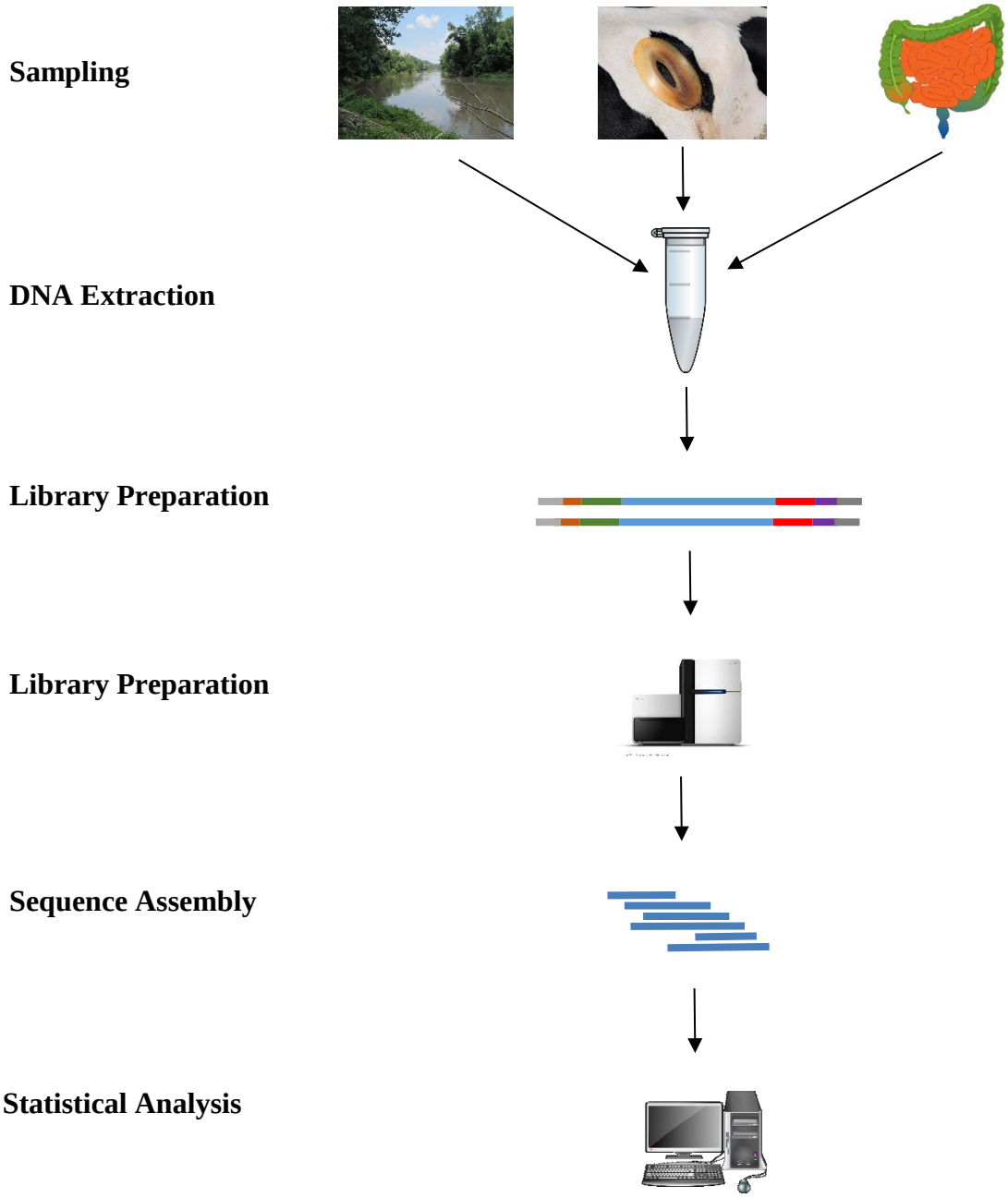
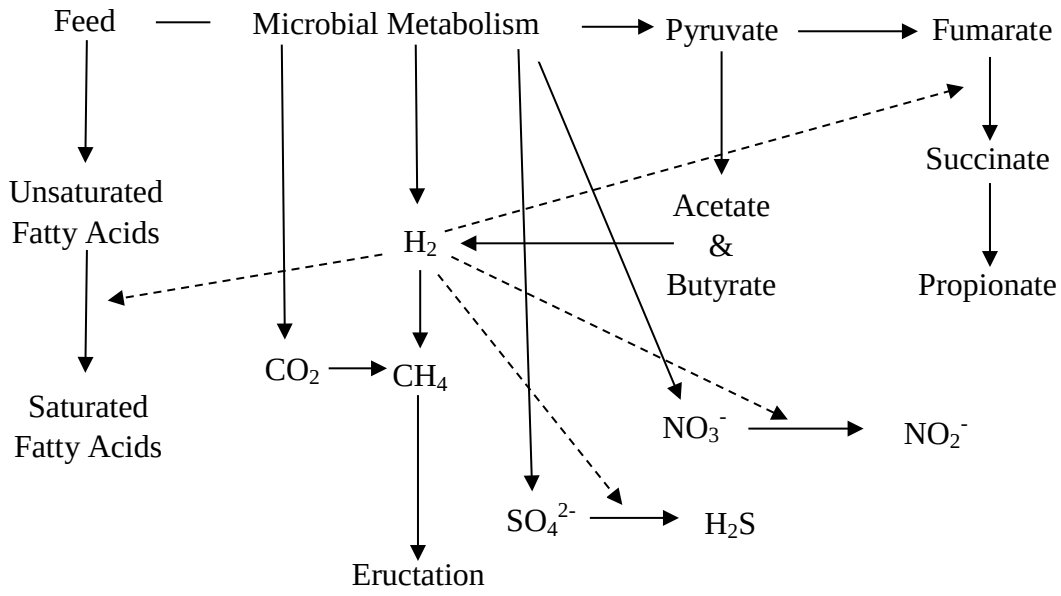


Figure 1.3. Mechanisms of rumen hydrogen production and disposal. Adapted from McAllister et al. (1996).



Effects of iso-alpha acids derived from *Humulus lupulus* (hops) extract on fermentation by rumen microbes in continuous culture fermenters

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SUMMARY

Iso-alpha acids from hops (*Humulus lupulus*) derived from the brewing industry have exhibited bacteriostatic properties against Gram-positive bacteria. Previous research demonstrated promising results for using whole or ground to decrease hyper-ammonia producing bacteria in the rumen. However, hops contain additional fermentable substrate and other metabolites including tannins, beta-acids and xanthohumol that confound the direct effects of iso-alpha-acids on rumen fermentation. Research on iso-alpha acids in rumen cultures is limited. The objective of this study was to examine direct effects of iso-alpha acids on fermentation by rumen microbes using a dual-flow continuous culture system. Eight fermenters were used in two consecutive 10 d periods consisting of 7 d of adaptation followed by 3 d of sampling. Fermenters were provided with a basal diet consisting of 44% corn silage, 14% alfalfa hay, 13% ground corn, 11% protein mix, 10% corn gluten feed, 5% cottonseed and 3% liquid vitamin and mineral supplements on a DM basis. This diet provided substrate for ruminal microbes maintained in continuous culture at a rate of 75 g of DM/L of fermenter volume/day. Iso-alpha extract (IE) solution was added to the artificial saliva buffer to supply 0 (CON), 600 (LOW), 1200 (MED) and 1800 (HIGH) mg of IE/kg of diet DM/day. There was no effect ($P > 0.05$) on DM, OM, NDF or ADF digestion (%). Volatile fatty acid (VFA) metabolism was not affected by IE treatment ($P > 0.05$), with total VFA concentrations of 105.5, 93.4, 87.9 and 103.6 mM for the CON, LOW, MED and HIGH treatments, respectively. Nitrogen metabolism was also not affected ($P > 0.05$) by IE level, with the CON, LOW MED, and HIGH treatments resulting in nitrogen concentrations of 7.4, 5.3, 7.6 and 6.8 mg N/dL of rumen fluid, respectively. No effects ($P > 0.05$) of treatment on fermenter pH were observed. In

conclusion, administration of IE had no impact on fermentation by ruminal microbes maintained in continuous culture fermenters.

Keywords: Rumen, Continuous Culture, Hops, Iso-Alpha Acids

INTRODUCTION

Ruminant animals have a uniquely adapted pregastric fermentation chamber, where symbiotic microbes convert cellulosic plant material and nonprotein nitrogen into volatile fatty acids (VFA) and high-quality microbial protein. The rumen allows the animal to utilize feed components that are otherwise unusable by non-ruminants. However, rumen fermentation also yields ammonia (NH_3) and methane (CH_4), nutritionally wasteful products that are associated with concerns over environmental pollution (Lana et al., 1998). Mitigation of these compounds by optimizing rumen ecology and fermentation is of great concern to producers of ruminant livestock.

Ionophore antibiotics are a commonly used dietary additive that can decrease NH_3 and CH_4 production in the rumen and improve animal efficiency by selective inhibition of specific microbial groups, mainly ciliated protozoa and gram-positive bacteria. However, there is increasing public concern regarding sub-therapeutic use of antibiotics in livestock production over their possible contribution to the emergence of antibiotic resistant bacteria (Narvaez et al., 2013a). As a result, there is an increasing interest in plant bioactive compounds as alternatives to ionophores for manipulating rumen fermentation.

Hops (*Humulus lupulus)* are one plant species that have demonstrated promise for use as a rumen fermentation modifier. Commonly used as a preservative and flavor enhancer in beer production, hops contain humulones (α-acids), lupulones (β-acids) and xanthohumol that exhibit bacteriostatic properties against gram positive bacteria (Haas and Barsoumian, 1994) and protozoa (Srinivasan et al., 2004) in mixed culture. These compounds are prenylated phloroglucinol derivatives found in the resin of hop flowers

which inhibit the growth of gram-positive bacteria through disruption of cell plasma membrane function (Keukeleire, 2000). This effect is likely caused by their ability to dissipate the transmembrane pH gradient, destroying the proton motive force across cells in a similar matter to ionophores (Sakamoto and Konings, 2003). More recent research results yielded evidence to suggest that the mechanism of hop antibacterial activity can be extended to uncoupling of redox reactions in the cellular membrane by iso-α-acids (Behr and Vogel, 2010).

Initial studies using spent hops showed mild effects on rumen fermentation including reductions in methane and acetic acid, as well as increases in propionic acid (Krishna et al., 1986). However, many of the antibiotic components of hops are lost during the brewing process, reducing some of their possible effects. In addition, the effects of whole hops on rumen fermentation both *in vitro* (Narvaez et al., 2011) and *in vivo* (Wang et al., 2010) have been assessed. While results using whole hop preparations are promising, results have been confounded by presence of additional fermentable matter and other antimicrobial compounds such as condensed tannins. Extraction of α and β-acids from hop resin, using supercritical CO_2 allows us the ability for more targeted research using these compounds. Hop extracts were found to decrease hyper ammonia-producing bacteria (HAB) in pure culture (Flythe, 2009) and decrease CH_4, ammonia nitrogen and acetate: propionate (A:P) ratio when hops extracts were administered in mixed batch culture (Narvaez et al., 2013a). Results have been inconsistent because other studies have showed little to no effect of hop extracts on rumen fermentation (Drouillard et al., 2009; Storlien et al., 2012; Fessenden et al., 2013, *unpublished data*). Therefore, future research must be conducted before conclusions can be made concerning

the efficacy of hops as feed additives in ruminants. The objective of this study was to determine the effects of iso-alpha extract from hop cones on *in vitro* fermentation by rumen microbes in continuous culture.

MATERIALS AND METHODS

Experimental Diet and Treatments

A basal diet was formulated to meet or exceed requirements of a Holstein cow producing 40 kg of milk/day with 2.8% fat and 3.7% protein (NRC, 2001). The basal diet consisted of 44% corn silage, 14% alfalfa hay, 13% ground corn, 11% protein mix, 10% corn gluten feed, 5% cottonseed and 3% liquid vitamin and mineral supplement on a DM basis. Ingredient and nutrient composition of the diet is provided in Table 2.1. After mixing, the diet was dried at 60°C in a forced air oven for 48 h and ground in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 2 mm screen. The ground diet was pelleted with a CL-5 California pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final pellet dimension of 6 mm diameter x 12 mm long. The pelleted diet was placed in shallow trays to air dry. Dry matter of the diet was measured on the day of inoculation and day 7 of both experimental periods by drying feed in an oven at 100°C for 24 hrs.

Hops iso-alpha extract was provided though treatment addition of the commercially available product Iso-Extract 30% (S.S. Steiner, Inc., New York, NY), which contained 30% (+/- 2%) iso-alpha extract in an aqueous solution, determined using high-pressure liquid chromatography. Iso-alpha extract (IE) was provided to the fermenters through the artificial saliva buffer in order to supply 0 (CON), 600 (LOW),

1200 (MED) and 1800 (HIGH) mg of iso-alpha acids/kg of diet DM/day. Artificial saliva that contained the treatments was freshly mixed daily. Iso-alpha extract stock solutions and artificial saliva solutions with IE were stored in the dark throughout the experimental period, to prevent breakdown of the iso-alpha acids prior to administration. Iso-alpha extract treatment concentrations were randomly assigned within each experimental period. Treatments were duplicated within each period to generate 4 experimental units per treatment.

Collection of Rumen Fluid Inoculum

The use of animals in this study was approved by the University of Minnesota Institutional Animal Care and Use committee (Protocol ID: 1304-30557A). Two ruminally cannulated lactating dairy cows served as rumen fluid donors. Composition of the diet fed to the donor cows was formulated to be the same as the experimental diet (Table 2.1). Rumen contents collected from each cow were collected and transported to the laboratory in pre-warmed thermoses. Contents from each cow were combined in equal parts and strained through 4 layers of cheesecloth. Strained rumen contents were homogenized and divided equally into 8 pre-warmed fermenters (1045 ± 30 mL per fermenter). Twenty-five grams of pelleted diet were added to fermenters immediately after inoculation.

Continuous Culture Operation

Eight continuous culture fermenters as described by Hannah et al. (1986), modified with a pH control and measurement system were used in two consecutive 10 d periods with 7 d of adaptation and 3 d of sampling. Pelleted feed was provided to the

fermenters at a rate of 75 g DM/L of fermenter volume/d. An automatic feeding system was used to deliver feed in 8 separate 90-minute intervals throughout the day. Artificial saliva buffer (pH=8.10) was prepared according to Weller and Pilgrim (1974) to provide a final concentration (g/L) of $NaHCO_3$, 5.0; Na_2HPO_4, 1.76; $KHCO_3$, 1.6; KCL, 0.6; $MgSO_4$, 0.05; and urea, 0.4. Liquid dilution rate for each fermenter was set to 10%/h by regulating the artificial saliva input while solids dilution rate was set at 5.5%/h by regulating liquid output through filters. Individual fermenter pH was measured continuously by an electronic data acquisition system (DASYLab v5.04, Measurement Computing, Norton, MA) and recorded every 5 minutes. Fermenter pH was maintained between 5.6 and 6.4 by automated addition of 5N NaOH or 3N HCL. Anaerobiosis was maintained within fermenters by addition of N_2 gas at a rate of 20 mL/min. Fermenter temperature was maintained at a temperature of 38.5 ± 0.1°C. Fermenter contents were agitated with a magnetic stir plate at 350 rpm.

Sample Collection

Samples were collected on the final 3 d of each 10-d period. Solids and liquid effluent samples were collected in separate vessels and maintained at 1°C in a water bath to reduce enzymatic and microbial activity. On sampling days, contents of solids and liquid effluent were combined within fermenter and homogenized using a PT10/3S homogenizer (Kinematica GmbH, Bohemia, NY). Five hundred mL of combined sample was collected daily from each fermenter during each of the 3 sampling days and composited by fermenter so that each sample contained effluent representing 3 d of collection in each period. A portion of the effluent sample was lyophilized for analysis of DM, OM, NDF, ADF, ash and purines. The remainder of the effluent was frozen and

subsequently thawed for analysis of VFA, N and NH_3-N. At the end of each 10 d experimental period, fermenter contents were filtered through 4 layers of cheesecloth and centrifuged at 1,000 x *g* to remove feed particles. Supernatant was then centrifuged at 20,000 x *g* to isolate microbial cells which were collected and lyophilized for analysis of DM, OM, total N and purines.

Chemical Analysis

Dry matter and ash content of the lyophilized effluent, lyophilized microbial cells and the experimental diet were determined by drying in an oven at 100°C for 24 h followed by combustion in a muffle furnace at 550°C for 24 h (AOAC 2005). Sequential detergent fiber analysis was conducted to determine NDF and ADF concentrations of diets and effluents using an ANKOM A200 fiber analyzer with F58 fiber bags (ANKOM Corp, Fairport, NY). Ammonia-N was determined on the supernatant of centrifuged (5,000 x *g*) effluent by steam distillation with magnesium oxide using a Kjeltec 2300 Analyzer Unit (Foss Tecator AB, Höganäs, Sweden). Total N of both effluent and diet were determined via the Kjeldahl method (AOAC, 1990). Purine concentration of effluents and microbial pellets were determined according to the procedure of Zinn and Owens (1986) with spectrometry being performed on a Synergy 2 Plate Reader with a Take3 Micro-volume plate (BioTek Instruments, Inc., Winooski, VT). The purine to N ratio used to determine flow of bacterial N and OM in the effluent samples.

Effluent VFA concentration was determined by gas chromatography. Prior to chromatography, fermenter effluent was clarified by centrifugation at 5,000 x *g* for 10 min. Supernatant was hydrolyzed using 25% meta-phosphoric acid, frozen overnight at -20°C and thawed, followed by additional centrifugation at 5,000 x *g* for 10 min to

remove hydrolyzed proteins. Clarified fluid was filtered through a 0.45 μm polyethersulfone micropore filter. Volatile fatty acid concentration was measured using an HP6890 gas chromatographer (Hewlett-Packard, Palo Alto, CA) with a 2m x 6.35mm x 2 mm Carbopack glass column (Supelco, Bellefonte, PA). Chromatograph conditions were as follows: injection volume-1.0 μL; injector temperature-200°C; carrier gas (N_2) flow rate-24.1 mL/min; flame ionization detector temperature-230°C. The procedure began with an initial oven temperature of 175°C which was held for 24 minutes. Oven temperature was then elevated by 25°C/min to 200°C and held for 4 min, followed by a post run temperature of 175°C for 4 min. Standardized solutions with known concentrations of VFA were analyzed to develop standard curves that were used to determine VFA concentrations of samples.

Statistical Analysis

All data processing and analyses were conducted using SAS software version 9.2 (SAS Institute, Inc., Cary, NC, USA). Data were analyzed as a randomized complete block design with experimental period serving as a block and all treatments equally represented within each block. The linear additive model for each dependent variable was:

$$Y_{ij} = \mu + P_i + A_j + (PA)_{ij} + e_{ij}$$

Where μ is the overall mean, P_i is the experimental period (block), A_j is the effect of iso-alpha acid level and $(PA)_{ij}$ is the interaction between period and acid and e_{ij} is the error term. For all analyses, differences were considered significant at $P \leq 0.05$, with a trend being described at $0.05 < P \leq 0.10$. Except for fermenter pH, all results were analyzed

using the GLM procedure of SAS. Differences between treatments were tested using LSMEANS with the PDIFF option in SAS. Results are reported as least squared means from 4 observations per treatment. Differences in treatments were reported as polynomial contrasts to determine linear and quadratic responses to IE inclusion. Dunnett's test was performed to compare the control to each IE level, however results are not presented because of non-significance.

Fermenter pH was recorded every 5 minutes during the 3 d sampling period and was summarized to determine simple mean, minimum, and maximum on an hourly basis. Repeated measures analysis was performed on the hourly averages using the MIXED procedure of SAS with a compound covariance structure. The model treated IE as a fixed effect and fermenter nested within period as a random effect. Time spent below pH 5.2, between pH 5.2 and 5.6 and above 5.6 were calculated using trapezoidal integration. Minutes were calculated from the raw dataset containing readings every 5 min. Comparisons between treatments were then conducted using the GLM procedure of SAS using LSMEANS with the PDIFF option.

RESULTS AND DISCUSSION

DM, OM and Fiber Digestion

Results for DM, OM and fiber digestion are presented in table 2.2. Iso-α-extract had no impact ($P > 0.10$) on true or apparent DM or OM digestion. There were also no differences ($P > 0.10$) in NDF or ADF digestion. Previous research on the effects of hops and hop extracts on diet digestion have been variable. Narvaez et al. (2011) observed an increase in *in vitro* apparent dry matter disappearance and *in vitro* true dry matter

disappearance (IVTDMD) when hops were added to rumen culture including barley silage or grain, and barley silage, respectively. Consistent with these results, Wang et al. (2010) found an increase in IVTDMD when whole hops were added to a barley-based TMR. Alternatively, Narvaez et al (2013b) and Lavrenčič (2014) reported decreases in IVTDMD when whole ground hops were included in culture. It is important to consider that studies using whole hops may have confounding effects of additional fermentable substrate and antimicrobial compounds other than IE, which may have contributed to the observed variations in results. Narvaez et al.(2013a) used hops extracts containing 22.5 μg/mL α-acids and observed a significant difference in IVTDMD, however the extract also contained β-acids which may have confounded results.

Volatile Fatty Acid Production

Volatile fatty acid concentrations and acetate to propionate ratio are presented in table 2.3. Total VFA concentration was not affected ($P > 0.10$) by IE inclusion and ranged from 87.9 to 105.5 mM. Likewise, molar proportions of individual VFA were not affected ($P > 0.10$) by IE treatment. Acetate to propionate ratio and branched-chain VFA (BCVFA) concentrations were also not affected ($P > 0.10$) by IE treatment. These results contrast with many previous *in vitro* studies using whole-hops or hops extract in rumen culture. Narvaez et al. (2011) discovered that ground whole hops decrease A:P ratio ($P < 0.001$) in rumen mixed culture with barley silage, barley grain or a TMR (Narvaez et al., 2011). Wang et al. (2010) determined in rumen batch culture that increasing ground hops concentrations linearly increased ($P < 0.01$) molar proportions of propionate and butyrate, while decreasing acetate production, and thus, A:P ratio ($P <0.01$). However, it is important to note that hops provided to these cultures were low in α-acid concentration

(11g/kg DM and 7 g/kg DM, respectively). Lavrenčič et al. (2015) studied the effects of two higher α-acid hop varieties using *in vitro* batch culture (115 g/kg DM and 147 g/kg DM) and detected a decrease ($P < 0.01$) in total VFA as well as acetate and butyrate molar proportions.

Using methanol-extracted hops extract containing 49.3 mg α-acids/g DM in rumen mixed culture, Narvaez et al. (2013a) observed an increase ($P < 0.01$) in molar proportions of propionate ($P < 0.01$) with decreases ($P < 0.01$) in butyrate and A:P ratio, which is in contrasts with results from the current experiment. Narvaez et al. (2013b) used a Rumen Simulation Technique (RUSITEC) system to study three different hop varieties. All three hops varieties increased ($P < 0.01$) molar proportions of propionate and A:P ratio. Interestingly, the high-α-acid varieties Millennium (116 g/kg DM) and Cascade (61.2 g/kg DM) reduced the molar proportion of butyrate, while the low α-acid variety Teamaker (7.7 g/kg DM) did not have this effect. Millennium hops also exerted a negative effect on BCVFA, while Teamaker exerted a positive effect. Their results may suggest a possible relationship between α-acid concentration of hops and butyrate and BCVFA, but this relationship was not confirmed in the current study. There are limited studies examining the effects of α-acids on VFA production *in vivo*. Al-Mamun et al. (2009) used isotope dilution to determine the effect of feeding whole-hops on acetate metabolism in sheep. They found no effect of hops on rumen production of total VFA or individual VFA molar proportions ($P > 0.10$). However, they observed a tendency ($P < 0.06$) for increased plasma acetate when hops were fed.

Nitrogen Metabolism

Ammonia-N concentration ranged from 5.3 to 7.6 mg/dL but was not significantly affected ($P > 0.10$) by IE inclusion (Table 2.4). The total flows of NH_3-N, non NH_3-N, microbial N, and dietary N, were not altered ($P > 0.10$) by IE treatment. IE inclusion also had no effect ($P > 0.10$) on CP degradation or efficiency of microbial protein synthesis. Flythe (2009) demonstrated a decrease in ruminal ammonia production and counts of hyper-ammonia producing bacteria when hop extracts were added to mixed rumen culture, but these extracts contained less than 1% α-acid, making it difficult to compare to the current study. Whole hops high in α-acids decreased CP degradation in batch culture using rumen fluid collected from sheep (Lavrenčič et al., 2014). An *in vivo* experiment by Al-Mamun et al. (2009) using cannulated sheep supplemented with hops observed a tendency for higher ruminal NH_3-N concentrations, but results may be confounded by the fact that diets varied in nitrogen concentration.

pH

Inclusion of iso-α-extract led to no effect on pH values within fermenters (Table 2.5). Mean, minimum, and maximum pH were not different ($P > 0.10$), nor was time below pH 5.8, between 5.8 and 6.2 and time above 6.2. Results of the present study are consistent with previous research using the RUSITEC system. Whole hops had no effects on fermentation pH (Narvaez et al., 2013b). Furthermore, ensiled hops elicited no effects on rumen pH in sheep (Al-Mamun et al., 2011). Addition of hop extracts to batch culture also had no effect on pH (Flythe and Aiken, 2010). Alternatively, changes in pH of batch culture medium in response to the inclusion of hops cannot accurately represent true

biological changes because these systems require a high buffering capacity to offset the accumulation of fermentations acids.

Iso-α-acid Inclusion Concentration

There are no published studies examining the direct effects of iso-α-acids on rumen fermentation in continuous culture. Therefore, previous literature could not be referenced to find a minimum effective dose of IE needed to elicit an effect on rumen fermentation. Inclusion rates for IE in the current study were chosen based on previous concentrations for β-acids used in continuous culture, with the aim of studying differences in responses of the two acid types (Fessenden et al., 2013, *unpublished data*). Because inclusion concentrations were not previously validated, they may be inadequate to elicit a response by rumen microorganisms. Narvaez et al. (2013b) supplied rumen culture with 0.05, 0.09, and 0.01 g of iso-α-acids per liter of fermenter volume per day from cascade, millennium, and teamaker hops, respectively. These iso-α-acid concentrations are far lower than those used in the current study which provided 45, 90, and 135 g iso-α-acids per liter of fermenter volume. Their study found reductions in total gas, methane and A:P ratio when hops were fed but results may have been due to the additional fermentable substrate provided from the whole hops, or from other antimicrobial effects within hops. Future studies should consider providing iso-α-acids at a higher inclusion concentration than that provided in the present experiment.

Microbial Adaptation

It is possible that the lack of a response from IE inclusion in continuous culture is due to adaptation by rumen microbes. In the brewing industry, several mechanisms of

bacterial resistance to hop acids have been described, including expression of multi-drug transporters and an energy conservation mechanism (Sami et al., 1997; Suzuki et al., 2002; Behr et al., 2007). Rumen microorganisms have exhibited resistance to antimicrobial feed additives, including monensin (Nagaraja and Taylor, 1987; Callaway et al., 1999). Monensin resistance appears to be conferred through development of extracellular polysaccharides that prevent ionophores from entering and disrupting the cell membrane (Russell and Houlihan, 2003). While rumen batch culture experiments demonstrated significant alterations in fermentation due to additions of hops and hops extract, limited effects have been demonstrated *in vivo*. Results from research demonstrated that the efficacy of antimicrobial compounds can be affected by adaptation time *in vivo* and in continuous culture, with as little as 6 days needed to reduce impacts on VFA production (Castillejos et al., 2007). Batch culture experiments typically use short-term incubations (< 72 hours), limiting the amount of time for the development of resistance processes and growth of resistant bacteria. In the current continuous culture experiment, rumen culture was allowed to stabilize for 7 days prior to sampling, which may have provided sufficient time for rumen microorganisms to adapt to the addition of iso-α-acids. Development of a resistance mechanism may partially explain the discrepancies between results from this study and other batch culture studies; however, no experimental evidence has been generated to support this conclusion. Future studies using hops in rumen culture would benefit from examination of changes in the gene expression of rumen microbes to determine if resistance development a factor contributing to lack of response by rumen microbes to hops inclusion *in vitro*.

CONCLUSIONS

Inclusion of iso-α-acids from hops yielded no effects on various measurements of fermentation in continuous culture. Estimates of digestion, VFA concentrations and nitrogen metabolism were within ranges previously observed in continuous culture studies (Hannah et al., 1986; Mansfield et al., 1995; Bach et al., 2008b). This study is the first known experiment to examine direct effects of iso-α-acids in continuous culture. Results indicate that inclusion of hops may not cause the same bacteriostatic effect in rumen culture that is observed when hops are used in the brewing industry. Inconsistencies may be due to differences in microbial community and metabolism between rumen microbes and microbes found within beer, too low an inclusion level or the development of microbial resistance mechanisms. Further research should be conducted to better elucidate the lack of response by rumen microbes to iso-α-acids.

Table 2.1. Ingredient and chemical composition of basal experiment diet.

Item	Composition[1]
Feed composition	
Corn silage	44.2
Alfalfa hay	13.5
Ground corn grain	12.6
Protein mix[2]	11.0
Corn gluten feed	9.6
Cottonseed	5.6
Liquid vitamin and mineral supplement[3]	3.4
Chemical composition	
Crude Protein	17.5
Soluble nitrogen (% of total N)	38.5
NDIN (% of total N)	11.7
ADIN (% of total N)	5.3
NDF	31.5
ADF	17.1
Lignin	3.2
Ash	7.4
Starch	26.4
Sugar	5.4
NFC	41.3
Crude fat	4.4
TDN	73.5
NE_L, 3X (Mcal/kg DM)	1.73

[1]Composition as % of DM unless otherwise noted.

[2]Protein mix composition (DM basis): canola meal, 28%; soybean meal, 22%; treated soybean meal, 15%; distillers dried grains, 13%; ground corn grain, 5%; calcium carbonate, 5%; bloodmeal, 3.5%; sodium bicarbonate, 3.5%; potassium carbonate, 3%; trace minerals, 2%.

[3]Supplement composition (DM basis): Ca, 49 g/kg; P, 11.7 g/kg; NaCl 101 g/kg; K, 34 g/kg; Mg, 7.2 g/kg; S, 5.5 g/kg; Mn, 1237 mg/kg; Cu, 382 mg/kg; Se, 8.6 mg/kg; Zn, 1813 mg/kg; Vitamin A, 171 IU/kg; Vitamin D, 34 IU/kg; Vitamin E, 706 IU/kg.

Table 2.2. Effects of increasing levels of hop iso-α-acid extract on DM, OM, and fiber digestion in continuous culture.

	Treatment[1]				SEM[2]	P-Value[3]		
Digestion (%)	**CON**	**LOW**	**MED**	**HIGH**		**Linear**	**Quadratic**	**Cubic**
DM, apparent	42.8	42.1	45.2	41.3	2.6	0.92	0.55	0.37
DM, true[4]	57.1	58.6	58.4	57.0	5.1	0.98	0.78	0.99
OM, apparent	38.9	37.9	41.6	37.3	2.4	0.92	0.52	0.27
OM, true[4]	51.4	52.4	53.0	51.0	4.8	0.98	0.76	0.92
NDF	36.4	35.7	43.0	34.4	4.8	0.96	0.43	0.29
ADF	42.4	39.5	48.0	39.5	4.1	0.99	0.51	0.15

[1]CON: 0 mg iso-α-acids/kg diet DM; Low: 600 mg iso-α-acids/kg diet DM; MED: 1200 mg iso-α-acids/kg diet DM; HIGH; 1800 mg iso-α-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic, and cubic contrasts.

[4]Corrected for bacterial contribution.

Table 2.3. Effects of increasing levels of hop iso-α-acid extract on VFA concentration in continuous culture.

	Treatment[1]				SEM[2]	P-Value[3]		
Volatile fatty acids	**CON**	**LOW**	**MED**	**HIGH**		**Linear**	**Quadratic**	**Cubic**
Total VFA, *mM*	105.5	93.4	87.9	103.6	17.9	0.89	0.46	0.86
Individual VFA, mol/100 mol								
Acetate	51.9	37.9	52.3	50.1	6.2	0.76	0.37	0.14
Propionate	28.6	22.4	28.4	31.2	5.4	0.58	0.42	0.54
Butyrate	14.3	11.2	14.3	13.7	2.2	0.91	0.60	0.35
Valerate	3.8	3.1	3.5	4.3	0.7	0.58	0.29	0.82
Isobutyrate	0.3	0.2	0.4	0.3	0.1	0.84	0.62	0.11
Isovalerate	0.2	0.1	0.4	0.2	0.1	0.57	0.79	0.09
2 Methylbutyrate	0.8	0.2	0.8	0.3	0.2	0.34	0.95	0.06
Branched-chain, *mM*	1.6	0.5	1.0	0.4	0.4	0.74	0.70	0.23
A:P Ratio	2.0	1.4	2.0	1.6	0.4	0.11	0.54	0.17

[1]CON: 0 mg iso-α-acids/kg diet DM; Low: 600 mg iso-α-acids/kg diet DM; MED: 1200 mg iso-α-acids/kg diet DM; HIGH; 1800 mg iso-α-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic, and cubic contrasts.

Table 2.4. Effects of increasing levels of hop iso-α-acid extract on nitrogen metabolism in continuous culture.

	Treatment[1]				SEM[2]	P-Value[3]		
Item	**CON**	**LOW**	**MED**	**HIGH**		**Linear**	**Quadratic**	**Cubic**
NH_3-N, mg/dL	7.4	5.3	7.6	6.8	2.0	0.95	0.76	0.42
N Flow, g/d								
NH_3-N	0.19	0.14	0.19	0.17	0.1	0.97	0.76	0.45
Non NH_3-N	2.07	2.10	1.86	2.20	0.1	0.79	0.26	0.17
Microbial N	0.94	1.05	0.86	1.05	0.3	0.93	0.90	0.66
Dietary N	1.05	1.05	1.00	1.15	0.3	0.98	0.68	0.88
CP degradation, %	58.1	60.5	63.3	57.2	9.4	0.97	0.67	0.83
EMPS[4]	39.5	38.2	31.8	44.4	9.2	0.84	0.47	0.57

[1]CON: 0 mg iso-α-acids/kg diet DM; Low: 600 mg iso-α-acids/kg diet DM; MED: 1200 mg iso-α-acids/kg diet DM; HIGH; 1800 mg iso-α-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic, and cubic contrasts.

[4]EMPS: efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested).

Table 2.5. Effects of increasing levels of hop iso-α-acid extract on fermentation pH in continuous culture.

	Treatment[1]					P-Value[3]		
pH	**CON**	**LOW**	**MED**	**HIGH**	**SEM[2]**	**Linear**	**Quadratic**	**Cubic**
Mean[4]	5.86	5.82	5.92	5.94	0.06	0.23	0.68	0.49
Minimum[4]	5.79	5.78	5.84	5.87	0.06	0.27	0.74	0.69
Maximum[4]	5.95	5.89	6.01	6.03	0.07	0.22	0.55	0.38
Time below 5.8[5]	1860.10	2663.53	1405.53	1400.39	605.41	0.35	0.52	0.24
Time between 5.8 and 6.2[5]	2354.90	1142.71	2461.66	2225.71	483.12	0.67	0.33	0.08
Time above 6.2[5]	102.49	510.01	450.31	691.41	256.12	0.16	0.75	0.52

[1]CON: 0 mg iso-α-acids/kg diet DM; Low: 600 mg iso-α-acids/kg diet DM; MED: 1200 mg iso-α-acids/kg diet DM; HIGH; 1800 mg iso-α-acids/kg diet DM.

[2]Standard error of the mean, n = 4 replicates per treatment.

[3]Probability corresponding to the null hypothesis with linear and quadratic, and cubic contrasts.

[4]Analyzed as repeated measures with 1 observation/fermenter/hour during 3 consecutive sampling days.

[5]Total minutes during 3 consecutive days, n = 4 replicates per treatment.

Comparisons of microbial populations found in the rumen and in a dual-flow continuous culture fermentation system using high-throughput 16S amplicon sequencing

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SUMMARY

16S amplicon sequencing was conducted to compare microbial communities found in the rumen of dairy cattle with that found in a dual-flow continuous culture fermentation system. Deoxyribonucleic acid (DNA) from rumen fluid samples, fermenter inoculum and fermenters were extracted and sequenced using the Illumina MiSeq platform. To assess the changes in microbial community during continuous culture fermenter operation, samples were collected from fermenters during each day of the experiment. Sequences were aligned using Mothur ver. 1.34.0 software and data were compared based on sample type (rumen vs. inocula vs. fermenter), inoculum donor and day of fermenter operation. Redundancy analysis (RDA) was performed to determine correlations between fermentation measurements based on microbial community. Correlations were also conducted to determine associations between prominent microbial families and fermentation measures from the *In vitro* system. Community differences were assessed using UniFrac metrics, Analysis of molecular variance (AMOVA) and Analysis of similarity (ANOSIM) based on Bray-Curtis dissimilarity matrices. Differences in taxonomic composition of different sample types were analyzed for kingdom phylum, class, order and family taxonomic levels. Functional inferences were made by matching taxonomic data to KEGG Orthology terms using PICRUSt software and analyzed based on sample type. Results showed that UniFrac, AMOVA and ANOSIM metrics were different ($P < 0.05$) between fermenters, and rumen and inoculum samples. Community profile did not differ ($P > 0.10$) between cows in either rumen or inoculum samples, but was different ($P < 0.05$) in fermenter samples. Microbial community within fermenters appeared to stabilize on day 7 of the experimental period according to AMOVA and ANOSIM analyses. *Bacteroidetes* and

Firmicutes made up the two most abundant phyla in rumen, inoculum and fermenters and neither group was different ($P > 0.10$) based on sample type. *Proteobacteria, Tenericutes, Spirochaetes* and *Verrucomicrobia* were different ($P < 0.05$) between sample types. Rumen, inoculum and fermenters did not differ ($P > 0.10$) in relative abundance of *Prevotellaceae*, which was the most abundant family in all three samples. Abundant families that were different ($P < 0.05$) by sample type included *Succinivibrionaceae, Lachnospiraceae* and *Paraprevotellaceae*. PICRUSt predictions showed that amino acid metabolism, membrane transport, energy metabolism and cellular processes and signaling were different ($P < 0.05$) between sample types. Metabolism of carbohydrates, cofactors and vitamins, and lipids were not affected ($P > 0.10$) by sample type according to PICRUSt inferences.

Keywords: Rumen, Continuous Culture, 16S Amplicon Sequencing, Functional Inferences

INTRODUCTION

The rumen is a complex microbial environment inhabited by a diverse consortia of bacteria, protozoa, archaea and fungi. These organisms impart a wide range of metabolic activity including degradation of the plant cell wall, synthesis of microbial protein from non-protein nitrogen sources such as ammonia and urea and biosynthesis of vitamins. The unique functions of rumen microorganisms have made ruminant animals economically vital components of global agriculture. The specific population of microbes present in the rumen can have a tremendous impact on extent and efficiency of nutrient digestibility. In turn, several factors can influence the rumen population, including nutrient composition of the diet, turnover rate, pH and presence of microbial-modifying feed additives in the diet (Russell, 2002). Studying the ecology of rumen microorganisms can provide tremendous insight into mechanisms affected by changes in the rumen environment.

Rumen fermentation studies can be aided by *in vitro* models that simulate the rumen microbial environment. Compared with *in vivo* models, *in vitro* methods are typically less expensive, less time consuming and more tightly controlled (Hristov et al., 2012). An *in vitro* system that adequately models the physical environment of the rumen must simulate *in vivo* pH, temperature, and turnover rates and maintain a core microbial population (Stern et al., 1997). The dual-flow continuous culture fermentation system developed by Hoover et al. (1976) has been shown to accurately model many important variables of rumen fermentation. This system has been found to closely estimate the effects of diet on NDF, ADF, and VFA concentrations, compared to *in vivo* values (Hoover et al., 1976). The first study to compare microbial populations between the

dual-flow system and the rumen of a cow was completed by Mansfield et al. (1995). This experiment revealed dramatic decreases in protozoa content in the fermenters compared with *in vivo* measurements, as well as increases in bacterial numbers. Concentrations of amylolytic and cellulolytic bacteria were lower in fermenters than *in vivo*, but proteolytic bacteria concentrations remained stable (Mansfield et al., 1995). While the experiment provided initial characterization of divergence between microbes in rumens and fermenters, the culture techniques used in the study suffer from cultivability bias. Zeimer et al. (2000) used 16S ribosomal RNA-targeted oligonucleotide probes to similarly compare microbial populations between the rumen and continuous culture fermenters. This experiment also observed decreases in protozoa and increases in total bacteria within the *in vitro* system, but found similar proportions of *Fibrobacter succinogenes*, *F. succinogenes* subgroup 3 and archaea between the two sample types (Ziemer et al., 2000). The authors concluded that continuous culture fermenters may be a suitable model to represent changes in certain microbial groups. This experiment was limited in its representation of the microbial community, however, on account of the narrow series of organisms examined.

Development of high-throughput genomic sequencing methods has greatly advanced the field of microbial ecology. These systems are able to rapidly generate large quantities of genomic information from a single sample, creating high resolution data on the microbial population contained in an environment. Whereas other techniques suffer from inherent bias or are limited in scope, high-throughput sequencing can provide a global assessment of all the organisms present in a sample. 16S amplicon sequencing of DNA obtained directly from an environmental sample has revolutionized the

investigation of the rumen microbial community (Denman and McSweeney, 2015). For example, use of these techniques has helped reveal changes in microbial populations associated with milk fat yield (Jami et al., 2014), host feed efficiency (McCann et al., 2014b) and rumen acidosis (Huo et al., 2014).

There has been no research to date using high-throughput 16S amplicon sequencing to explore microbial populations in continuous culture fermenters. If a core population of organisms is maintained within fermenters, they could serve as a model to study shifts in microbial communities when provided various dietary substrates or antimicrobial feed additives. The main objective of this experiment was to use high-throughput 16S amplicon sequencing to compare microbial populations of the rumen and dual-flow continuous culture system, to determine the extent of difference between core microbial communities and to examine which taxa differed between the two models. Moreover, the fluctuations in microbial populations over the course of fermenter operations, and the effects of inoculum donor on microbial population within continuous culture fermenters were also examined within this experiment.

MATERIALS AND METHODS

Experimental Diets and Treatments

Cows and fermenters were fed a basal diet formulated to meet or exceed the requirements of a Holstein cow producing 40 kg of milk/day with 2.8% fat and 3.7% protein (NRC, 2001). The experimental diet contained approximately 50% corn silage, 14% protein mix, 13% alfalfa haylage 8% ground corn, 4% corn gluten, 3% molasses and 2% cottonseed (Table 3.1). Diets were mixed and dried in a forced air oven at 60°C for

48h. After drying, the diet was ground in a Wiley No. 4 laboratory mill (Arthur H. Thomas Co., Philadelphia, PA) to pass through a 2-mm screen, and pelleted in a CL-5 California pellet mill (California Pellet Mill Co., Crawfordsville, IN) to a final pellet dimension of 6 mm diameter x 12 mm long. Pellets were placed in shallow trays and dried at room temperature for 48 h. DM of the pellets was measured on days 0, 5 and 7 of both experimental periods.

Cows and Rumen Fluid Collection

Two multiparous lactating dairy cows were fitted with rumen cannulas according to the guidelines set by the University of Minnesota Animal Care Committee (Protocol ID: 1304-30557A). Cows were adapted to the basal diet for 21 d prior to rumen fluid collection. Rumen samples were manually collected in 50 mL conical tubes at four time points during the sampling day at 0, 2, 4 and 6 h post-feeding. To ensure a representative microbial community, samples were collected from the cranial and caudal regions of the ruminoreticulum. Approximately 5 L of rumen inoculum from each cow were separately collected into pre-warmed thermoses and transported back to the laboratory. Contents from cows were strained through 4 layers of cheesecloth into two separate thermoses and homogenized. Inoculum from each cow was sampled into two 50 mL conical tubes and immediately frozen at -40°C for deoxyribonucleic acid (DNA) extraction. Eight pre-warmed fermenters (1048 ± 28 mL per fermenter) were randomly assigned to receive inoculum from one of the two cows, with 4 fermenters being inoculated per cow. Twenty-five grams of pelleted diet were added to the fermenters immediately after inoculation.

Continuous Culture Operation

Eight continuous culture fermenters as described by Hannah (1986), modified with a pH control and measurement system were used in two consecutive 10 d periods with 7 d adaptation and 3 d of sampling. Pelleted feed was provided to the fermenters at a rate of 75 g DM/L of fermenter volume/d. An automatic feeding system delivered substrate in eight separate 90 minute intervals throughout the day. Artificial saliva buffer (pH = 8.29) was prepared according to Weller and Pilgrim (1974) to provide a final concentration (g/L) of $NaHCO_3$, 5.0; Na_2HPO_4, 1.76; $KHCO_3$, 1.6; KCL, 0.6; $MgSO_4$, 0.05; and urea, 0.4. Liquid flow rate for each fermenter was set to 10%/h by regulating the artificial saliva input, while solids dilution rate was set at 5.5%/h by regulating liquid output through filters. Individual fermenter pH was measured continuously by an electronic data acquisition system (DASYLab v13.0, Measurement Computing, Norton, MA) and recorded every 15 seconds. Fermenter pH was maintained between 5.6 and 6.4 by automated addition of 5N NaOH or 3 N HCL. Anaerobiosis was maintained within fermenters by the addition of N_2 gas at a rate of 20 mL/min. Fermenter temperature was maintained at 38.5 ± 0.1°C. Fermenter contents were agitated with a magnetic stir plate at 350 rpm.

Sample Collection

Solid and liquid effluent samples were collected from each fermenter at 0900, 1100 and 1300 each day of the study. Thirty mL of solid and liquid effluent were collected at each time point, totaling 90 mL for each fraction collected per fermenter per day. Samples were immediately frozen at -20°C after collection to prevent bacterial lysis. Daily effluent samples were stored at -40°C until DNA extraction.

On the final 3 d of each 10 d experimental period, total solid and liquid effluent was collected into separate vessels maintained at 1°C in a water bath to reduce enzymatic and microbial activity. At 1430 on each of the 3 sampling days, solid and liquid effluents were collected, combined within fermenter, and homogenized using a PT10/3S homogenizer (Kimetica GmbH, Bohemia, NY). Five hundred mL of combined sample were collected daily from each fermenter during each of the 3 sampling days and composited by fermenter so that each sample contained effluent representing 3 d of collection in each period. A portion of the effluent sample was lyophilized for analysis of DM, OM, NDF, ADF, ash and purines. The remainder of the effluent was frozen and subsequently thawed for analysis of VFA, N and NH_3-N. At the end of each 10 d experimental period, fermenter contents were filtered through 4 layers of cheesecloth and centrifuged at 1,000 x *g* to remove feed particles. Supernatant was then centrifuged at 20,000 x *g* to isolate microbial cells which were collected and lyophilized for analysis of DM, OM, total N and purines.

DNA Extraction

Daily solid and liquid effluent samples were thawed and 16.5 mL of solid, and 13.5 mL of liquid effluent were combined and mixed thoroughly using a pipet-aid. These volumes were chosen to match the 55:45 ratio of solid:liquid effluent leaving the fermenters. Seven point five mL aliquots of rumen fluid from each of the 4 time points were aggregated and mixed. Rumen fluid, inoculum and fermenter effluent samples were centrifuged at 1000 x *g* in a swinging-head centrifuge to separate solid feed particles from liquid-associated bacteria. Supernatant was centrifuged at 4000 x *g* to isolate the bacterial pellet. Supernatant was discarded and DNA was extracted from the microbial

cells using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA), which uses bead beating with sodium dodecyl sulfate to lyse microbial cell membranes, followed by cycles of inhibitor solution addition and centrifugation to remove proteins and other particulates. Quantity and quality of DNA were assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Rumen fluid and inoculum sample nucleic acid concentrations averaged 102 ng/μL. Nucleic acid concentration in fermenter samples averaged 71 ng/μL. Deoxyribonucleic acid quality was assessed spectrophotometrically by $A_{260nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ ratios. Rumen and inoculum samples had an averaged 1.86 and 1.22 for $A_{260nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ ratios, respectively. Fermenter effluent generated an average $A_{260nm}/A_{280\ nm}$ ratio of 1.86 and an average $A_{260\ nm}/A_{230\ nm}$ratio of 1.44. These values are similar to those previously reported in the literature (Kang et al., 2009; Popova et al., 2010; Henderson et al., 2013).

DNA Sequencing and Sequence Processing

The V4 hypervariable region of the 16S rRNA gene was PCR amplified using the 515F/806R barcoded primer set with KAPA HiFidelity Hot Start Polymerase. Pooled, size-selected samples were denatured with NaOH, diluted to 8 picometers in Illumina's HT1 buffer, spiked with 15% PhiX, and heat denatured at 96°C for 2 minutes immediately before loading into the sequencer. Replicate sequence data were generated using pair-end sequencing of purified amplicon pools using Illumina MiSeq (2 x 300 read length) (Illumina, Inc., San Diego, California, U.S.). Library preparation and sequencing were performed by the University of Minnesota Genomics Center.

Sequence processing was performed using Mothur software ver. 1.34.0 (Schloss et al., 2009). Sequences were trimmed to 100 nucleotides, paired-end aligned using fastq-join (Aronesty, 2013) and screened for quality. Sequences were excluded from analysis if they had a quality score less than 35 over a window of 50 nucleotides, a mismatch to a primer or barcode sequence, homopolymers that were greater than 8 nucleotides or at least one ambiguous base. Singleton sequences were removed in mothur and chimeras were removed using UCHIME (Edgar et al., 2011). The number of sequence reads in each sample was normalized by random subsampling to 30,000 sequence reads per sample. Sequences were aligned against the SILVA database ver. 119 (Quast et al., 2012). Operational taxonomic units (OTUs) were clustered using the furthest-neighbor algorithm at 97% similarity and OTUs were classified against the Ribosomal Database Project ver. 14. Functional predictions were made based on the KEGG Orthology (KO) database using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software package ver. 1.0.0 (Langille et al., 2013). To compare functional estimates among samples, data were normalized as a percentage of the total number of predicted functions from the KO database. Comparisons between samples were made on tier 2 KO assignments.

Chemical Analysis

Dry matter and ash content of the lyophilized effluent and microbial cells, and the experimental diet were determined by drying in an oven at 100°C for 24 h followed by combustion in a muffle furnace at 550°C for 24 h (AOAC 2005). Sequential detergent fiber analysis was conducted to determine NDF and ADF concentrations of diets and effluents using an ANKOM A200 fiber analyzer with F58 fiber bags (ANKOM Corp,

Fairport, NY). Ammonia-N was determined on the supernatant of centrifuged (5,000 x *g*) effluent by steam distillation with magnesium oxide using a Kjeltec 2300 Analyzer Unit (Foss Tecator AB, Höganäs, Sweden). Total N of both effluent and diet were determined via the Kjeldahl method (AOAC 1990). Purine concentration of the effluent and microbial pellet were determined according to the procedure by Zinn and Owens (1986) with spectrometry being performed on a Synergy 2 Plate Reader with a Take3 Micro-volume plate (BioTek Instruments, Inc., Winooski, VT). The purine to N ratio in effluents and microbial cells was used to determine flow of bacterial N and OM in the effluent.

Effluent VFA concentrations were determined using gas chromatography. Prior to chromatography, fermenter effluent was clarified by centrifugation at 5,000 x *g* for 10 min. Supernatant was hydrolyzed using 25% meta-phosphoric acid, frozen overnight at -20°C and thawed, followed by an additional centrifugation at 5,000 x *g* for 10 min to remove hydrolyzed proteins. Clarified fluid was filtered through a 0.45 μm polyethersulfonemicropore filter. Volatile fatty acid (VFA) concentration was measured using an HP6890 gas chromatographer (Hewlet-Packard, Palo Alto, CA) with a 2 m x 6.35 x 2 mm Carbopack glass column (Supelco, Bellefonte, PA). Chromatograph conditions were as follows: injection volume – 1.0 μL; injector temperature - 200°C; carrier gas (N_2) flow rate- 24.1 mL/min; flame ionization detector temperature- 230°C. The procedure began with an initial oven temperature of 175°C which was held for 28 minutes. Oven temperature was then elevated by 25°C/min to 200°C and held for 4 min, followed by a post-run temperature of 175°C for 4 min. Standardized solutions with known concentrations of VFA were analyzed to develop standard curved that were used

to determine VFA concentrations of samples. 2-ethylbutyric acid was used an internal standard to adjust for injection volume.

Statistical Analysis

Organic Matter, DM, fiber, crude protein digestibility, VFA production, individual VFA concentrations and nitrogen flows, and pH were analyzed as a randomized block design with experimental period serving as a block and inoculum donor equally represented within block using the GLM procedure of SAS 9.2 (SAS Institute, Inc. Cary, NC, USA). Fermentation pH recorded every 15 seconds obtained over 3 d of sampling was summarized to determine simple mean, minimum, and maximum on an hourly basis. Repeated measures analysis was performed on the hourly averages using the MIXED procedure of SAS with a compound covariance structure. The model treated inoculum donor as a fixed effect and fermenter nested within period as a random effect. Time spent below pH 5.2, between pH 5.2 and 5.6, and above 5.6 were calculated using trapezoidal integration. Minutes were calculated from the raw dataset containing readings every 15 seconds. Data were analyzed using the GLM procedure of SAS using LSMEANS with the PDIFF option.

All community comparisons and their respective statistical analyses were performed using Mothur (Schloss et al., 2009) and Bray-Curtis dissimilarity matrices (Bray and Curtis, 1957). For all analyses, replicates were treated as separate samples and grouped according to variable of interest using separate design files. Operational taxonomic unit data were analyzed based on sample type (cow vs. inoculum vs. fermenter), inoculum source (cow), and day of fermenter operation. Three analyses were used to evaluate differences in community structure based on the variables of interest: (i)

UniFrac metrics, which calculate the relative relatedness of community members by incorporating raw (unweighted) or abundance-weighted (weighted) phylogenetic distances between sets of taxa in different samples (Lozupone and Knight, 2005); (ii) analysis of similarity (ANOSIM), which calculates beta-diversity using rank order differences in community structure based on a dissimilarity matrix (Clarke, 1993); and (iii) analysis of molecular variance (AMOVA), which is similar to a non-parametric analysis of variance using the molecular variation of OTUs (Excoffier et al., 1992). Principal component analysis (PCoA) was performed to determine the clustering pattern of the samples. Briefly, PCoA uses orthogonal transformation to reduce the number of original variables, in this case OTUs, into a smaller number of uncorrelated variables, called *principal components* (Reich et al., 2008). The distance among different phylogenetic communities can be visualized by plotting the principal components against one another. A three-dimensional PCoA plot was created using the EMPeror visualization tool (Vázquez-Baeza et al., 2013).

Redundancy analysis (RDA) was conducted using *rda()* function in the R package vegan 2.3.0 (Oksanen et al., 2011). Briefly, RDA uses constrained ordination to determine the amount of variation in one variable that is explained by another variable. In the present experiment, the variation in fermentation measurements between was predicted using the variation in in microbial families. The RDA was plotted to determine the strength of correlations between measures of nutrient digestibility, VFA concentrations, nitrogen metabolism, and pH. Spearman correlations (ρ) and their associated significance tests were calculated between fermentation measurments using the *rcorr()* function in the R package Hmisc. The *rcorr()* function was also used to

calculate Spearman correlations between fermentation measurements and relative abundances of prominent microbial groups.

The MIXED procedure of SAS 9.2 was used to determine statistical differences in OTU abundance between sample types at five taxonomic levels (kingdom, phyla, class, order and family) and for PICRUSt functional predictions. Fixed effects included sample type and period and type by period interaction. Mean OTU abundances and functional inferences were separated by sample type using the LSMEANS function with the PDIFF option. Statistical significance was determined at $P < 0.05$, while a trend was recognized at $0.10 > P \geq 0.05$ for all data.

RESULTS AND DISCUSSION

Fermentation Parameters

Digestion of OM, DM, fiber and crude protein, VFA production, individual VFA concentrations, nitrogen flows and pH were analyzed to confirm that operation of continuous culture fermenters was similar to previous studies and to determine if inoculum donor affected fermentation measurements. For all fermenters, apparent and true DM digestibility averaged 41.3 and 55%, respectively, while apparent and true OM digestion averaged 31.7 and 44.1%, respectively. Inoculum donor had no effect ($P > 0.10$) on true or apparent DM or OM digestion. Acid detergent fiber and NDF digestion were also not affected ($P > 0.10$) by inoculum donor (Table 3.2).

Means for total VFA, A:P ratio and BCVFA were 99.5 mM, 1.73, 0.91, respectively (Table 3.3). Total VFA, branched chain VFA (BCVFA), individual VFA molar proportions and A:P ratio were not altered ($P > 0.10$) by inoculum source. Total

NH_3-N averaged 8.1 mg/dL, crude protein degradation averaged 66.7 and efficiency of microbial protein synthesis (EMPS) averaged 29.6 g microbial of N/kg of OM truly digested. Inoculum donor exhibited no effect ($P > 0.10$) on total N, daily N flows, CP degradation, or EMPS (Table 3.4). Mean, minimum, and maximum fermentation pH were not affected ($P > 0.10$) by inoculum donor, nor were time below pH 5.8, time between pH 5.8 and 6.2, or time above pH 6.2 (Table 3.5). All fermentation parameters were within ranges previously observed in continuous culture studies (Hannah et al., 1986; Mansfield et al., 1995; Bach et al., 2008a).

Redundancy analysis RDA was used to determine correlations between fermentation parameters based on microbial ecology present within a sample (Figure 3.1). Unsurprisingly, RDA showed strong correlations ($P < 0.01$) between OM and DM digestion ($\rho = 0.89$), NDF and ADF digestion ($\rho = 0.89$) and NH_3 concentration and NH_3-N flow ($\rho = 0.94$). Total VFA concentration exhibited a low ($P > 0.10$) correlation with acetate concentration ($\rho = 0.09$), propionate concentration ($\rho = 0.13$), butyrate ($\rho = 0.02$), BCVFA ($\rho = 0.13$) and A:P ratio ($\rho = -0.07$). There was a strong negative relationship ($P < 0.01$), between acetate and propionate concentrations ($\rho = -0.89$) and subsequently strong relationships between A:P ratio and both acetate ($\rho = 0.94$), and propionate ($\rho = -0.99$) concentrations. Low correlations ($P > 0.10$) was observed between EMPS and both CP degradation ($\rho = 0.32$) and NH_3 concentration ($\rho = -0.24$).

Spearman correlations and their subsequent significant tests were conducted to correlate the relative abundance of microbial families with fermentation measurements (Table 3.6). DM digestion was correlated ($P < 0.05$) with the abundance of *Spirochaetaceae*, and tended to be correlated ($P < 0.10$) with *Veillonellaceae* and

Ruminococcaceae. Organic matter digestion was correlated ($P < 0.05$) with *Spirochaetaceae* and *Ruminococcaceae* relative abundance while exhibiting a trend ($P < 0.10$) for correlation with relative abundance of "Other", *Paraprevotellaceae,* and *Alteromondaceae*. The *Spirochaetaceae Ruminococcaceae* and *Veilonellaceae* families contain many well-described bacterial species known to be important for ruminant digestion. Both NDF and ADF digestion demonstrated a trend ($P < 0.10$) for correlation with *Succinivibrionaceae* and *Erysipelotrichaceae*, and ADF digestion was also correlated ($P < 0.05$) with *Desulfovibrionaceae* and tended ($P < 0.10$) to be correlated with *Veillonellaceae*. Additionally, *Desulfovibrionaceae* and *Veillonellaceae* displayed correlations ($P < 0.05$) with CP degredation, NH_3 concentration, and NH_3-N daily flow. A trend ($P < 0.10$) for correlation between NH_3 concentration and NH_3-N flow with *Succinivibrionaceae* were observed, as well as a trend ($P < 0.10$) for correlation with NH_3 concentration and "Other" microbes. Efficiency of microbial protein synthesis was shown to correlate ($P < 0.05$) with *Veilonellaceae, Ruminococcaceae,* and *Spirochaetaceae*, and showed a tendency ($P < 0.10$) for correlation with *Desulfovibrionaceae* abundance. Total VFA and F16 were revealed to be correlated ($P < 0.05$) and a trend was observed between *Desulfovibrionaceae* and Total VFA.

Community Comparisons

Variation in community composition based on day of fermenter operation, sample type (rumen vs. inoculum vs. fermenter) and inoculum donor was assessed by PCoA and AMOVA (Table 3.7). Overall, there was an effect ($P < 0.05$) of experimental day on community profile. The PCoA plot (Figure 3.2) revealed that rumen and inoculum samples clustered together, which was confirmed by similar ($P > 0.10$) molecular

variation assessed by AMOVA. Lack of disparity between rumen an inoculum indicates little change occurred in the microbial community during collection, transport or filtration of inoculum. Rumen samples were composites of four sampling times from the cow throughout the day, and inoculum was collected at a single time point immediately before inoculation of the fermenters. Average pH decreased from 6.15 in rumen fluid to 5.56 in the inoculum. Results demonstrated that despite differences in sampling time and pH, little molecular variation was present between these samples. The intent of the current study was to isolate only liquid phase bacteria by centrifugation of feed particles prior to DNA extraction because inclusion of particle-associated bacteria in extracted DNA could lead to a greater difference in microbial community between rumen and inoculum because of lack of fiber particles in inoculum after filtration.

The PCoA plot showed that day 0 samples exhibited a unique clustering pattern that slightly overlapped with rumen and inoculum samples. AMOVA revealed differences ($P < 0.05$) between rumen and day 0 fermenter samples, but not ($P > 0.10$) between day 0 and inoculum. Day 0 represented the first partial day (18 h) after fermenter inoculation. Lack of change between inoculum and day 0 could be attributed to the short time within the *in vitro* system. Distinct clustering patterns were observed for samples from days 0 and 1 in the PCoA plot, supported by a tendency for difference ($P < 0.10$) in molecular variance between the two days. Results proposed that a greater change in microbial population occurred after the first full day of fermenter operation than immediately after inoculation. Additional clusters were observed for days 2, 3 and 4, but substantial overlapping of PCoA data points was observed on days 5 through 10. AMOVA analysis established that beyond days 0 and 1, there was no difference ($P >$

0.10) in community structure between back-to-back days. Community structure was comparable ($P > 0.10$) between samples from day 6 to day 10, indicating stabilization of microbial population within fermenters.

AMOVA was used to determine the effect of inoculum donor on the microbial community within rumen, inoculum and fermenter samples from days 8 through 10 of operation. Samples collected before day 8 were excluded to ensure that the microbial population had fully stabilized and to maintain consistency with the sampling period for non-microbial analyses. There was no molecular variance ($P > 0.10$) between donors for rumen or inoculum samples, but inoculum donor did exert an effect ($P < 0.05$) within fermenter samples. These results are surprising considering that inoculum donor did not influence molecular variance in rumen or inoculum samples. Furthermore, fermentation measurements were not impacted by inoculum donor. Because of these other observations, it is difficult to suggest that inoculum donor truly affected fermenter microbial population. The difference detected could be due to the high number of replicates producing false significance. Further research should be performed to confirm or deny the difference determined in the current study. Confounding due to inoculum donor is typically not a problem in continuous culture studies because inoculum is usually collected from one singular cow (Ariza et al., 2001; Castillejos et al., 2007; Bach et al., 2008b) or composited from two cows (Calsamiglia et al., 2002; Ruiz-Moreno, 2012; Fessenden et al., 2013).

Weighted and unweighted UniFrac metrics were used to assess differences in phylogenetic community based on sample type and inoculum donor. Unweighted UniFrac for the overall effect of sample type was 0.99, indicating a disparate ($P < 0.05$)

phylogenetic profile. Weighted UniFrac compared phylogenetic profile between each pair of sample types (Table 3.8). Scores were dissimilar ($P < 0.05$) between rumen and fermenters and between inoculum and fermenters, but exhibited a trend ($P < 0.10$) between rumen and inoculum samples. Unweighted and weighted UniFrac scores based on inoculum donor were 0.94 and 0.74, respectively, which both indicated a distinction ($P < 0.05$) between donors. Previous research conducted by Jami and Mizrahi (2012) compared weighted UniFrac metrics across samples from 16 lactating dairy cows fed the same diet. They observed little difference in weighted UniFrac metrics between samples collected from individual cows, although significance was not reported. The results of the current study suggest that phylogenetic distance may differ between individual cows fed the same diet, but conclusions are difficult to make because of the small number of rumen samples.

Beta diversity was compared between sample types and inoculum donors using ANOSIM. Similar to AMOVA, only fermenter samples from the sampling period (d 8 to 10) were used for these comparisons. Beta diversity was comparable ($P > 0.10$) between rumen and inoculum samples. These results are similar to outcomes from AMOVA and UniFrac, reinforcing the notion that microbial community does not change from rumen sampling, transport of inoculum to the laboratory, or filtration through cheesecloth. ANOSIM revealed differing ($P < 0.05$) beta diversity between fermenters and inoculum and between fermenter and rumen samples, which is consistent with UniFrac results. There was no difference ($P > 0.10$) in beta diversity between inoculum donors for either rumen or inoculum samples. Conversely, beta diversity varied ($P < 0.05$) between inoculum donors within fermenter samples. The discrepancy in beta diversity between

inoculum donors within fermenters is similar to observations from AMOVA, and is likely to be caused by the large number of fermenter samples rather than inoculum source.

ANOSIM was also used to calculate beta diversity between samples based on day of fermenter operation (Table 3.9). No difference ($P > 0.10$) in beta diversity was observed between fluid or inoculum and samples from days 0 or 1 of fermenter operation. Beta diversity was dissimilar ($P < 0.05$) between days 0 and 1, but no difference ($P > 0.10$) was discovered between any other adjacent days. Sample beta diversity appeared to stabilize on day 7 because days 7, 8, 9 and 10 had similar ($P > 0.10$) ANOSIM measurements. Results were comparable to those of AMOVA which suggested that molecular variance stabilized by day 6 of operation. Continuous culture fermenter studies have typically used a 5 to 7 day adaptation period prior to a three-day sampling period (Hoover et al., 1976; Mansfield et al., 1995; Calsamiglia et al., 2002; Ruiz-Moreno, 2012). Ziemer et al. (2000) reported that total 16S rRNA, and relative abundances of bacteria, eukaryotes, archaea, and *Fibrobacter* did not change ($P > 0.10$) after 96 h (4 d) of fermenter operation. However, the study examined a limited number of taxa and did not fully characterize diversity within a sample. Results from the current study indicate that a minimum of 6 days of adaptation is needed for the microbial population to stabilize.

Taxonomic Data

Comparisons between relative abundances of operational taxonomic units (OTUs) were performed between rumen, inoculum and fermenter samples at five taxonomic levels: Kingdom, Phylum, Class, Order and Family. Only fermenter samples from days 8 through 10 were included in the OTU abundance comparisons to avoid confounding

effects of experiment day. The V4 primer used in the study allowed us to capture the gene sequences of bacteria and archaea in the samples. As expected, bacteria were more abundant than archaea, with greater than 98% of sequences identified as bacteria in all three sample types (Table 3.10). Archaea made up between 0.22 and 0.26% of the assigned OTUs, with remaining sequences unable to be assigned. Overall, bacteria demonstrated a tendency ($P < 0.10$) for differences between sample types. Rumen samples tended ($P < 0.10$) to have a greater bacterial abundance than fermenters, but inoculum did not differ ($P > 0.10$) between fermenter or rumen samples. Archaeal abundance was unaffected ($P > 0.10$) by sample type.

At the phylum level, *Bacteriodetes* and *Firmicutes* were the first and second most abundant groups present in all three sample types (Figure 3.3). These observations are consistent with previous characterizations of rumen phyla from cattle (Kong et al., 2010; Kim et al., 2011; Jami et al., 2013). *Bacteroidetes* and Firmicutes were both found at similar ($P > 0.10$) abundances in all sample types. *Proteobacteria*, typically found at the third highest abundance in the rumen, was dissimilar ($P < 0.05$) between sample types and was found at a lower ($P < 0.05$) abundance in inoculum samples than fermenter samples. Samples types differed ($P < 0.05$) in other abundant phyla including *Tenericutes*, *Spirochaetes* and *Verrucomicrobia*. *Cyanobacteria* and TM7 were not impacted ($P > 0.10$) by sample type.

Bacteroidia and *Clostridia* were the most abundant classes in all three samples types (Figure 3.4). Differences ($P > 0.10$) in mean abundance of either class were not found between sample types. *Gammaproteobacteria* was as found at the third highest level in rumen and fermenter samples, but not inoculum. Sample type influenced ($P <$

0.05) *Gammaproteobacteria* abundance, with a disagreement ($P < 0.05$) between fermenter and inoculum samples, but with rumen and inoculum or fermenters. *Mollicutes*, "Other", *Spirochaetes*, *Verruco-5* and *Erysipelotrichi* were other abundant classes that were found at discordant ($P < 0.05$) levels between sample types. At the order level, *Bacteriodales*, and C*lostridiales* were the most predominant groups in all three sample types and neither class was affected ($P > 0.10$) by sample type (Figure 3.5). Orders that were influenced ($P < 0.05$) by sample type include: *Aeromandales*, "Other", *Spirochaetales*, *Erysipelotrichales* and WCHB1-41.

At the family level, *Prevotellaceae* was the most abundant group in all three sample types, and relative abundance was not different ($P > 0.10$) by sample type (Figure 3.6). The *Prevotella* genus is the most prominent member of the *Provotellaceae* family found in the rumen, and includes many well-described species including *Prevotella ruminicola and Prevotella bryantii* (Avgustin et al., 1997). Results from the current experiment are similar to previous research using 16S-based qPCR which revealed that *Prevotella* was the dominant genus within the rumen (Stevenson and Weimer, 2007). This genus carries many metabolically important functions, particularly digestion of non-cellulosic carbohydrates and production of succinate (Purushe et al., 2010). *Prevotella* are also known to be proteolytic and have the capability of amino acid deamination (Russell, 2002). Many members of the *Prevotella* genus have been noted for their resistance to ionophore antibiotics (Newbold et al., 1993; Callaway and Russell, 1999). The results of the current imply little change in abundance of *Prevotellaceae* between natural rumens and dual-flow continuous culture fermenters, proposing that they may be accurately modeled in this particular *in vitro* system.

The second and third most abundant families were identified as "Other" and *Succinivibrionaceae* ($P < 0.05$). The "Other" category was found at divergent ($P < 0.05$) levels between samples, with rumen samples having a greater ($P < 0.05$) relative abundance than fermenter samples. Organisms classified as "Other" are unable to be identified within the SILVA database (Quast et al., 2012). The fact that unidentified organisms make up the second most abundant family in the current study indicates that further research must be done to better characterize rumen microbial genome sequences. The *Succinivibrionaceae* family was also found at differing levels ($P < 0.05$) between sample types, with fermenter samples having higher ($P < 0.05$) relative abundance than inoculum samples. *Succinivibronaceae* includes amylolytic species *Ruminobacter amylophilus* and *Succinomonas amylolytica*, as well as maltodextrin-degrading *Succinivibrio dextrinosolvens*. Previous research in continuous culture fermenters has demonstrated greater ($P < 0.05$) digestion of total non-structural carbohydrates (TNC) in continuous culture compared with *in vivo* measurements, which may be related to the increase in *Succinivibrionaceae* observed in the present study (Mansfield et al., 1995). This increase in *Succinivibrionaceae* may be related to the well-described decrease in protozoa within continuous culture fermenters (Hoover et al., 1976; Mansfield et al., 1995; Ziemer et al., 2000). Amylolytic bacteria species were shown to be greater in defaunated rumens and may also increase with the low-protozoa conditions of the rumen (Nagaraja et al., 1992).

Veillonellaceae constituted between 5.03 to 8.33% of total OTU abundance but did not differ ($P > 0.10$) between sample types. Three well-characterized species within the *Veillonellaceae* family include: *Anaerobivibrio lipolytica*, *Megasphaera elsdenii*, and

Selenomonas ruminantium, all three of which are lactate-utilizers (Mackie and Heath, 1979). Lactate utilization has been studied extensively using continuous culture, with focus on populations of lactate-producing and lactate-utilizing bacteria (Russell and Baldwin, 1979; Russell and Dombrowski, 1980; Russell et al., 1981). The present study provides evidence that continuous culture may be an appropriate model to study changes in populations of these organisms. *Streptococcaceae*, the family that contains well-known lactate producer *Streptococcus bovis*, was found at a very low abundance in all three sample types. However, it is interesting to note that its relative abundance was not different ($P > 0.10$) between sample types, and was almost numerically identical between rumen (0.0051%) and inoculum (0.0052%) samples.

Other prominent families that were altered ($P < 0.05$) by sample type included *Lachnospiraceae*, *Paraprevotellaceae*, C*omamonadaceae*, *Spirochaetaceae*, *Erysipelotrichaceae*, *RF16* and *RFP12*. The *Ruminococcacae* family, which includes prominent fiber-degrading bacteria *Ruminococcus albus* and *Ruminococcus flavefaciens* was not affected ($P > 0.10$) by sample type. On the other hand, *Fibrobacteraceae*, which contains the well-described cellulose-degrader *Fibrobacter succinogenes*, was nearly absent *in vitro*. Relative abundance of *Fibrobacteraceae* was reduced ($P < 0.05$) from 0.33% in the rumen, to only 0.08% in inoculum and 0.02% in fermenters. This deviation could be attributed to the scarcity of long fiber particles in inoculum and fermenter samples after filtration through cheesecloth. *F. succinogenes* exhibits complex fiber-adherence mechanisms which cause it to be highly associated with the plant cell wall and be far less prevalent in the in the liquid phase (Jun et al., 2007). The *Methanobactericae* family which contains all identified rumen methanogens, was not affected ($P > 0.10$) by

sample type despite a numerical increase from 0.09% in rumen samples to 0.20% in inoculum and 0.24% in fermenters. The numerical increase observed in the current study was similar to results observed by Ziemer et al (2000) who found an greater relative abundance of methanogenic archaea in continuous culture fermenters than *in vivo*.

While many important microbial families did not differ between rumen and fermenter samples, it is important to interpret these results with caution. The current study used rumens and fermenters provided a single dietary treatment and fermenters had limited pH and flow rate ranges. Interactions may exist between diet, turnover rate, and pH which could influence the relationship between *in vivo* and *in vitro* abundances of taxonomic groups. Further research should be done to examine relationships between rumens and fermenters under different fermentation conditions. The present experiment provides baseline measures for microbial abundances within continuous culture fermenters in future studies with similar diet, pH and turnover rate.

Unfortunately, the analysis used in the current study did not allow determination of OTU abundances at the genus or species level. Current high-throughput sequencing technologies are able to produce deep coverage of samples but are limited in their read length, which prohibits accurate taxonomic identification of taxa below the family level (Mizrahi-Man et al., 2013). Furthermore, these technologies are not yet able to accurately characterize protozoa, which are important members of the rumen microbial community (Hu et al., 2015). As DNA sequencing technologies continue to advance, longer read lengths will allow better characterization the rumen microbiome.

Functional Inferences

Normalized level 1 and level 2 functional inferences were compared based on sample type. Among level 1 KO terms, the greatest number of genes (~ 50%) encoded proteins involving "metabolism" in all three sample types (Table 3.10). "Metabolism" was followed by "genetic information processing" (~22%), "unclassified" (~14%), and "environmental information processing" (9 – 11%). Of the 8 assigned KO terms, only "metabolism", and "environmental information processing" were affected ($P < 0.05$) by type. Means separation revealed a difference ($P < 0.05$) in "metabolism" assigned genes between fermenter and both rumen and inoculum samples, but not ($P > 0.10$) between rumen and inoculum.

A total of 38 level 2 KO terms were matched to samples using PICRUSt (Table 3.11). Because of the large number of KO terms matched, only functions that are metabolically important in the rumen will be discussed in this paper. "Amino acid metabolism", "carbohydrate metabolism", "replication and repair', and "membrane transport" were the most frequently matched terms in all samples. "Amino acid metabolism" was affected ($P < 0.05$) by sample type with fermenter samples having less predicted function than both rumen and inoculum samples. Discordant with this finding, neither "carbohydrate metabolism" nor "replication and repair' were impacted by sample type ($P > 0.10$). PICRUSt predictions for carbohydrate metabolism differ from observations by Mansfield et al. (1995), who detected differences in TNC and NDF digestion between fermenters and *in vivo*. Discrepancies between previous measures of rumen fermentation and predicted functions may be due to generality of the "carbohydrate metabolism" KEGG term, or indicate limitations in PICRUSt's ability to

infer function in the rumen environment. Other non-microbial factors that not present in our study may have also contributed to differences in carbohydrate metabolism between *in vivo* and *in vitro* models within the Mansfield et al. (1995) study, which could partially explain the contrasting results.

"Membrane transport" was altered ($P < 0.05$) by sample type, with differences ($P < 0.05$) between fermenter and inoculum samples. Several antimicrobial feed additives work through disruption of cell membrane transport (Bergen and Bates, 1983; Nagaraja and Taylor, 1987; Wallace, 2004). The results of the present study suggest that changes in functional inferences observed after addition of antimicrobial feed additives may not reflect *in vivo* conditions. Other functions that were divergent ($P < 0.05$) between treatments included "energy metabolism", "cellular processes and signaling", "biosynthesis of secondary metabolites" and "transport and catabolism". All these functions differed ($P < 0.05$) between fermenters and both rumen and inoculum samples ($P < 0.05$), but not between rumen and inoculum. Several metabolically important terms including "metabolism of cofactors and vitamins", "glycan biosynthesis and metabolism", "lipid metabolism", and "metabolism of terpenoids and polyketides" did not vary ($P > 0.10$) between treatments.

There are many limitations to PICRUSt functional inferences as an approach for determining the functional capabilities of a microbial ecosystem. The PICRUSt algorithm relies on a reference set of fully-sequenced microbial genomes to predict functions of other unsequenced organisms, causing its accuracy to be reliant on the number of fully sequenced microbes in a community (Langille et al., 2013). PICRUSt should be used with caution when analyzing functional predictions from novel or highly

diverse communities. For example, low correlations were observed between functional profiles obtained with PICRUSt and shotgun metagenomic analysis in bacterial communities obtained from the upper Mississippi River (Staley et al., 2014). Genomic sequences of rumen microbes are still somewhat limited, making functional predictions from the rumen less reliable than predictions from more well-characterized communities such as the human microbiome (Turnbaugh et al., 2007). Projects such as Hungate1000, which aims to sequence full genomes of 1000 prominent rumen microbial species, will help improve the ability to make functional predictions (Creevey et al., 2014). Presently, there are 271 described genomes in the Hungate1000 database, with another 234 genomes currently in progress (http://www.hungate1000.org.nz/). Because of the limitations of PICRUSt software, it is important that data from this study be treated as estimates of function and not considered with as much weight as other analytical measurements. True profiling of gene expression in a microbial sample must be performed using deep metagenomic sequencing across many samples, which is prohibitively expensive for most applications (Langille et al., 2013).

CONCLUSIONS

The current study is the first to compare microbial populations of rumens and dual-flow continuous culture fermenters using high-throughput 16S amplicon sequencing. Results indicated that overall community profiles deviated between rumen and fermenter samples but not between rumen and inoculum. Although overall community changed, the relative abundance of the two most prominent phyla, *Bacteroidetes* and *Firmicutes* did not vary between samples. There was also no divergence between relative abundances of *Prevotellaceae* in the three sample types.

However, several other metabolically important taxa were present at dissimilar abundances in rumens and fermenters. Functional predictions from PICRUSt software implied differences in amino acid metabolism, membrane transport, and energy metabolism, and other microbial activities. Antithetically, there were no distinctions in carbohydrate, lipid, or vitamin and cofactor metabolism between sample types. The current study maintains that fermenters may be an appropriate model for investigating differences in relative abundance of certain microbial groups and metabolic functions. Future research should be conducted to better illustrate changes in microbial populations of continuous culture fermenters fed differing substrates or alternative at pH levels. The present study provides baseline OTU abundance and PICRUSt functional prediction values for future studies on microbial ecology in continuous culture fermenters with similar fermentation conditions.

Table 3.1. Ingredient and chemical composition of basal experiment diet.

Item	Composition[1]
Feed composition	
Corn silage	48.5
Protein mix[2]	13.7
Alfalfa haylage	12.3
Ground corn grain	7.6
Corn gluten feed	4.2
Molasses	3.3
Cottonseed	2.2
Alfalfa Hay	1.7
Grass Hay	1.6
Energy Booster	0.8
Chemical composition	
Crude Protein, %	16.1
Undegraded CP (RUP), % of CP	38.8
Degraded CP (RDP), % of CP	61.2
Soluble CP, % of CP	33.0
NDF, %	29.9
ADF, %	19.1
NDFd, %	45.7
Ash	
Starch, %	26.3
Sugar, %	4.4
NFC, %	42.5
Crude fat, %	5.1
TDN, %	74.3
NE_L, Mcal/kg DM	0.78

[1]Composition as % of DM unless otherwise noted.

[2]Protein mix composition (DM basis): fine rolled corn, 37%; soybean mean, 15%; canola meal, 12%; treated soybean meal, 10%; distillers dried grains, 8%; blood meal, 5%; calcium carbonate, 4%; sodium bicarbonate, 4%; trace minerals, 2%; protected methionine, 2%; salt, 2%; potassium carbonate, 1%; urea, 1%.

Table 3.2. Effects of inoculum donor on DM, OM and fiber digestion in continuous culture.

	Inoculum Donor			
Digestion (%)	**Cow 1**	**Cow 2**	**SEM**[1]	**P-Value**[2]
DM, apparent	44.0	38.6	3.0	0.22
DM, true[3]	57.2	53.7	3.4	0.48
OM, apparent	33.9	29.4	2.5	0.22
OM, true[3]	45.5	42.6	2.9	0.51
NDF	26.3	23.0	4.7	0.63
ADF	31.6	29.4	4.5	0.74

[1]Standard error of the mean n=8 replicates per cow.

[2]Probabilty corresponding to the null hypothesis.

[3]Corrected for bacterial contribution.

Table 3.3. Effects of inoculum donor on volatile fatty acid concentration in continuous culture.

	Inoculum Donor			
Volatile fatty acids	**Cow 1**	**Cow 2**	**SEM[1]**	**P-Value[2]**
Total VFA, m*M*	95.9	103.1	9.4	0.60
Individual VFA, mol/100 mol				
Acetate	44.4	51.8	4.7	0.29
Propionate	26.7	28.6	3.3	0.69
Butyrate	12.1	14.7	1.6	0.27
Valerate	3.5	3.9	0.42	0.50
Isobutyrate	0.26	0.32	0.06	0.47
Isovalerate	0.17	0.23	0.08	0.58
2 Methylbutyrate	0.50	0.54	0.19	0.90
Branched-chain, m*M*	0.69	1.13	0.29	0.31
A:P Ratio	1.52	1.94	0.27	0.30

[1]Standard error of the mean n=8 replicates per cow.

[2]Probabilty corresponding to the null hypothesis.

Table 3.4. Nitrogen metabolism by inoculum donor in continuous culture.

Item[1]	Inoculum Donor		SEM[1]	P-Value[2]
	Cow 1	Cow 2		
NH_3-N, mg/dl	7.9	8.4	0.8	0.63
N flow, g/d				
NH_3-N	0.18	0.19	0.02	0.71
Non NH_3-N	1.78	1.84	0.05	0.37
Microbial-N	0.91	0.93	0.08	0.87
Dietary-N	0.87	0.91	0.07	0.67
CP degradation, %	67.5	65.9	2.5	0.74
EMPS[3]	27.9	31.4	3.8	0.53

[1]Standard error of the mean n=8 replicates per cow.

[2]Probabilty corresponding to the null hypothesis.

[3]Efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested).

Table 3.5. Fermentation pH by inoculum donor in continuous culture.

pH	Inoculum Donor		SEM[1]	P-Value[2]
	Cow 1	Cow 2		
Mean[3]	5.77	5.75	0.02	0.36
Minimum[3]	5.72	5.69	0.02	0.16
Maximum[3]	5.83	5.82	0.03	0.86
Time below 5.8[4]	2755.35	2912.38	176.80	0.54
Time between 5.8 and 6.2[4]	1502.68	1375.97	179.52	0.63
Time above 6.2[4]	49.48	19.14	25.41	0.41

[1]Standard error of the mean n=8 replicates per cow.
[2]Probabilty corresponding to the null hypothesis.
[3]Analyzed as repeated measures with 1 observation/fermenter/hour during 3 consecutive sampling days.
[4]Total minutes during 3 consecutive sampling days.

Table 3.6. Spearman correlations between fermentation measurements and relative abundances of prominent microbial families.[1, 2]

	Fermentation Measurement								
	DM dig.	OM dig.	NDF dig.	ADF dig.	CP deg.	NH_3	NH_3-N	EMPS	Total VFA
Prevotellaceae	-0.16	-0.15	0.24	0.06	0.29	-0.23	-0.20	0.28	0.17
Succinivibrionaceae	-0.27	-0.16	-0.47	-0.44	0.36	-0.47	-0.48	0.41	0.07
Other	0.38	0.49	0.20	0.22	-0.04	0.45	0.32	-0.33	0.35
Veillonellaceae	0.46	-0.30	-0.36	-0.46	0.56	-0.76	-0.68	0.64	0.12
Ruminococcaceae	0.48	0.61	0.39	0.29	-0.11	0.21	0.05	-0.64	0.40
Lachnospiraceae	0.06	0.30	-0.15	-0.13	0.14	-0.29	-0.32	-0.01	0.15
Paraprevotellaceae	0.32	0.43	0.36	0.26	-0.09	0.19	0.11	-0.36	0.27
S24-7	-0.06	-0.11	0.04	0.11	0.13	0.15	0.15	0.25	0.09
RF16	0.24	0.23	-0.01	0.08	-0.32	0.41	0.39	-0.42	-0.02
F16	0.24	0.34	0.22	0.24	0.27	0.19	0.11	-0.03	0.51

[1]Data reported as Spearman correlations with correlations, with significant ($P < 0.05$) correlations displayed in red, correlations exhibiting a trend ($0.05 < P < 0.10$) displayed in yellow, and those showing non-significance ($P > 0.10$) displayed in white/grey.

[2]Key: OM dig. = true organic matter digestion (%), DM dig. =true dry matter digestion (%), NDF dig. = neutral detergent fiber digestion (%), ADF dig. = acid detergent fiber digestion (%), CP deg. = crude protein degradation (%),NH_3= ammonia concentration (mg/dl), NH_3-N = Ammonia nitrogen flow(g/d), EMPS= Efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested) Total VFA= total volatile fatty acid concentration (m*M*), Acetate= acetate concentration (mol/100 mol), Propionate= concentration (mol/100 mol), BCVFA = branched-chain volatile fatty acid concentration (m*M*), A:P= acetate:propionate ratio.

Table 3.6. Spearman correlations between fermentation measurements and relative abundances of prominent microbial families (*continued from pg. 109)*.[1, 2]

	Fermentation Measurement								
	DM dig.	OM dig.	NDF dig.	ADF dig.	CP deg.	NH_3	NH_3-N	EMPS	Total VFA
Alteromonadaceae	-0.24	-0.46	-0.22	-0.21	-0.10	0.03	0.19	0.27	-0.42
Comamonadaceae	-0.10	0.16	-0.18	-0.36	0.24	-0.37	-0.41	0.06	0.17
Acidaminobacteraceae	-0.14	-0.36	-0.31	-0.20	-0.31	0.36	0.42	-0.03	-0.42
Lactobacillaceae	0.00	0.11	-0.32	-0.27	0.24	-0.19	-0.22	-0.05	0.23
Erysipelotrichaceae	-0.29	-0.11	-0.46	-0.47	0.20	-0.31	-0.37	0.17	0.29
Spirochaetaceae	0.53	0.55	0.33	0.36	-0.10	0.19	0.10	-0.53	0.20
Desulfovibrionaceae	-0.34	-0.13	-0.34	-0.50	0.68	-0.58	-0.67	0.48	0.48
Methanobacteriaceae	0.01	-0.11	-0.13	-0.14	-0.14	0.06	0.09	0.02	-0.32

[1]Data reported as Spearman correlations with correlations, with significant ($P < 0.05$) correlations displayed in red, correlations exhibiting a trend ($0.05 < P < 0.10$) displayed in yellow, and those showing non-significance ($P > 0.10$) displayed in white/grey.

[2]Key: OM dig. = true organic matter digestion (%), DM dig. =true dry matter digestion (%), NDF dig. = neutral detergent fiber digestion (%), ADF dig. = acid detergent fiber digestion (%), CP deg. = crude protein degradation (%),NH_3= ammonia concentration (mg/dl), NH_3-N = Ammonia nitrogen flow(g/d), EMPS= Efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested) Total VFA= total volatile fatty acid concentration (m*M*), Acetate= acetate concentration (mol/100 mol), Propionate= concentration (mol/100 mol), BCVFA = branched-chain volatile fatty acid concentration (m*M*), A:P= acetate:propionate ratio.

Table 3.7. Community changes assessed by AMOVA.[1, 2]

				Experimental Day									
		Rumen	Inoculum	0	1	2	3	4	5	6	7	8	9
	Inoculum	0.566											
Experimental Day	0	0.033	0.129										
	1	0.066	0.070	0.079									
	2	0.005	<0.001	<0.001	0.210								
	3	0.001	<0.001	<0.001	0.040	0.301							
	4	<0.001	<0.001	<0.001	0.010	0.031	0.480						
	5	<0.001	<0.001	<0.001	0.003	0.014	0.049	0.707					
	6	0.001	<0.001	<0.001	<0.001	0.004	0.049	0.350	0.765				
	7	<0.001	<0.001	<0.001	0.002	<0.001	0.004	0.072	0.232	0.970			
	8	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	0.012	0.251	0.442	0.671		
	9	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	0.017	0.019	0.295	0.798	0.157	
	10	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	0.016	0.155	0.471	0.249	0.988

Overall Model

F-stat: 3.18 P-Value: < 0.001

[1]Data presented as P-values for differences between sample types and experimental days.

[2]Sample types/days different ($P < 0.05$) are presented in red, those exhibiting a trend ($0.10 > P \geq 0.05$) are presented in yellow, and those not different ($P > 0.10$) are presented in green.

Table 3.8. Weighted UniFrac scores between pairs of sample types.

Sample Pair	WScore[1]	P-Value[2]
Rumen-inoculum	0.69	<0.001
Inoculum-fermenter	0.89	<0.001
Rumen- fermenter	0.95	0.062

[1]Weighted UniFrac score corresponding to the phylogenetic distance between sample types.

[2]Probability that weighted UniFrac score indicates a difference in phylogenetic community between sample types.

Table 3.9. Community changes assessed by ANOSIM.[1, 2]

				Experimental Day									
		Rumen	Inoculum	0	1	2	3	4	5	6	7	8	9
	Inoculum	0.664											
Experimental Day	0	0.163	0.521										
	1	0.206	0.288	0.048									
	2	0.018	0.015	<0.001	0.209								
	3	0.001	<0.001	<0.001	0.036	0.346							
	4	0.001	<0.001	<0.001	0.003	0.041	0.503						
	5	0.003	<0.001	<0.001	0.002	0.012	0.046	0.677					
	6	0.005	<0.001	<0.001	0.004	0.006	0.045	0.338	0.754				
	7	0.004	0.003	<0.001	<0.001	<0.001	0.003	0.050	0.285	0.963			
	8	0.001	<0.001	<0.001	0.001	<0.001	0.002	0.003	0.346	0.542	0.673		
	9	0.004	<0.001	<0.001	<0.001	0.005	0.003	0.010	0.005	0.283	0.742	0.119	
	10	0.002	<0.001	<0.001	0.001	<0.001	0.002	0.003	0.003	0.081	0.412	0.140	0.949

[1]Data presented as P-values for differences between sample types and experimental days.

[2]Sample types/days different ($P < 0.05$) are presented in red, those exhibiting a trend ($0.10 > P \geq 0.05$) are presented in yellow, and those not different ($P > 0.10$) are presented in green.

Table 3.10. Relative abundance of kingdoms between sample types.

Kingdom	Sample Type			F-Value	P-Value[3]
	Rumen[1]	Inoculum[1]	Fermenter[2]		
Bacteria	98.72 ± 0.22	99.09 ± 0.16	99.11 ± 0.05	2.59	0.08
Unassigned[4]	1.02 ± 0.07[a]	0.68 ± 0.18[ab]	0.64 ± 0.04 [b]	3.62	0.03
Archaea	0.26 ± 0.12	0.22 ± 0.09	0.25 ± 0.03	0.03	0.97

[1]Data presented as means ± standard error of the mean with n=4 samples per type.
[2]Data presented as means ± standard error of the mean with n=48 samples per type.
[3]Probabilty corresponding to the null hypothesis.
[4]Sample types with different superscripts in a row are different at $P < 0.05$.

Table 3.11. Difference in level 1 PICRUSt Functional Inferences between sample types.

Item[1]	Sample Type[2]			P-Value[4]
	Rumen[2]	Inocula[2]	Fermenter[3]	
Metabolism[5]	50.23 ± 0.35[a]	50.29 ± 0.19[a]	49.20 ± 0.14[b]	0.006
Genetic Information Processing	22.08 ± 0.05	22.14 ± 0.13	21.82 ± 0.07	0.344
Unclassified	13.70 ± 0.01	13.71 ± 0.03	13.76 ± 0.02	0.479
Cellular Processes	2.47 ± 0.07	2.52 ± 0.08	2.54 ± 0.03	0.848
Environmental Information Processing	9.75 ± 0.34	9.57 ± 0.31	10.92 ± 0.17	0.010
Organismal Systems	0.81 ± 0.02	0.82 ± 0.03	0.79 ± 0.01	0.231
Human Diseases	0.79 ± 0.01	0.77 ± 0.01	0.79 ± 0.01	0.369
None	0.18 ± 0.00	0.18 ± 0.00	0.18 ± 0.00	0.409

[1]Level 1 KEGG Orthology terms as predicted by PICRUSt.
[2]Data is presented as means ± standard error of the mean with n=4 samples per type.
[3]Probabilty corresponding to the null hypothesis.
[4]Data is presented as means ± standard error of the mean with n=48 samples per type.
[5]Sample types with different superscripts in a row are different at $P < 0.05$.

Table 3.12. Differences in level 2 PICRUSt functional inferences between sample types.

Item[1]	Sample Type			P-value[4]
	Rumen[2]	Inocula[2]	Fermenter[3]	
Amino acid metabolism[3]	10.44 ± 0.08[a]	10.46 ± 0.03[a]	10.11 ± 0.03[b]	<0.001
Carbohydrate metabolism	10.37 ± 0.05	10.34 ± 0.01	10.19 ± 0.05	0.361
Replication and repair	10.23 ± 0.03	10.26 ± 0.10	10.12 ± 0.04	0.382
Membrane transport[3]	8.44 ± 0.32[ab]	8.27 ± 0.26[a]	9.42 ± 0.16[b]	0.007
Translation	6.74 ± 0.06	6.75 ± 0.02	6.56 ± 0.02	0.034
Energy metabolism[3]	6.23 ± 0.06[a]	6.21 ± 0.02[a]	5.95 ± 0.03[b]	0.001
Poorly characterized	4.76 ± 0.01	4.78 ± 0.00	4.80 ± 0.01	0.735
Metabolism of cofactors and vitamins	4.71 ± 0.06	4.70 ± 0.04	4.72 ± 0.03	0.839
Nucleotide metabolism	4.58 ± 0.04	4.61 ± 0.04	4.53 ± 0.02	0.162
Cellular processes and signaling[3]	3.81 ± 0.05[a]	3.80 ± 0.03[a]	3.99 ± 0.01[b]	<0.001
Glycan biosynthesis and metabolism	3.04 ± 0.09	3.07 ± 0.10	3.04 ± 0.06	0.816
Folding, sorting and degradation	2.77 ± 0.02	2.75 ± 0.02	2.75 ± 0.02	0.966
Lipid metabolism	2.73 ± 0.01	2.73 ± 0.02	2.77 ± 0.02	0.464
Genetic information processing	2.67 ± 0.02	2.68 ± 0.02	2.72 ± 0.01	0.170
Metabolism[3]	2.48 ± 0.04[a]	2.48 ± 0.01[a]	2.32 ± 0.01[b]	<0.001
Transcription	2.38 ± 0.02	2.41 ± 0.02	2.46 ± 0.02	0.088
Enzyme families	2.24 ± 0.02	2.27 ± 0.03	2.23 ± 0.01	0.237
Metabolism of terpenoids and polyketides	1.82 ± 0.02	1.83 ± 0.02	1.80 ± 0.01	0.311
Cell motility	1.52 ± 0.09	1.57 ± 0.08	1.59 ± 0.04	0.503
Metabolism of other amino acids	1.59 ± 0.02	1.58 ± 0.01	1.54 ± 0.01	0.961
Xenobiotics biodegradation and metabolism	1.50 ± 0.02	1.52 ± 0.02	1.56 ± 0.02	0.699
Signal transduction	1.16 ± 0.03	1.14 ± 0.05	1.22 ± 0.02	0.115

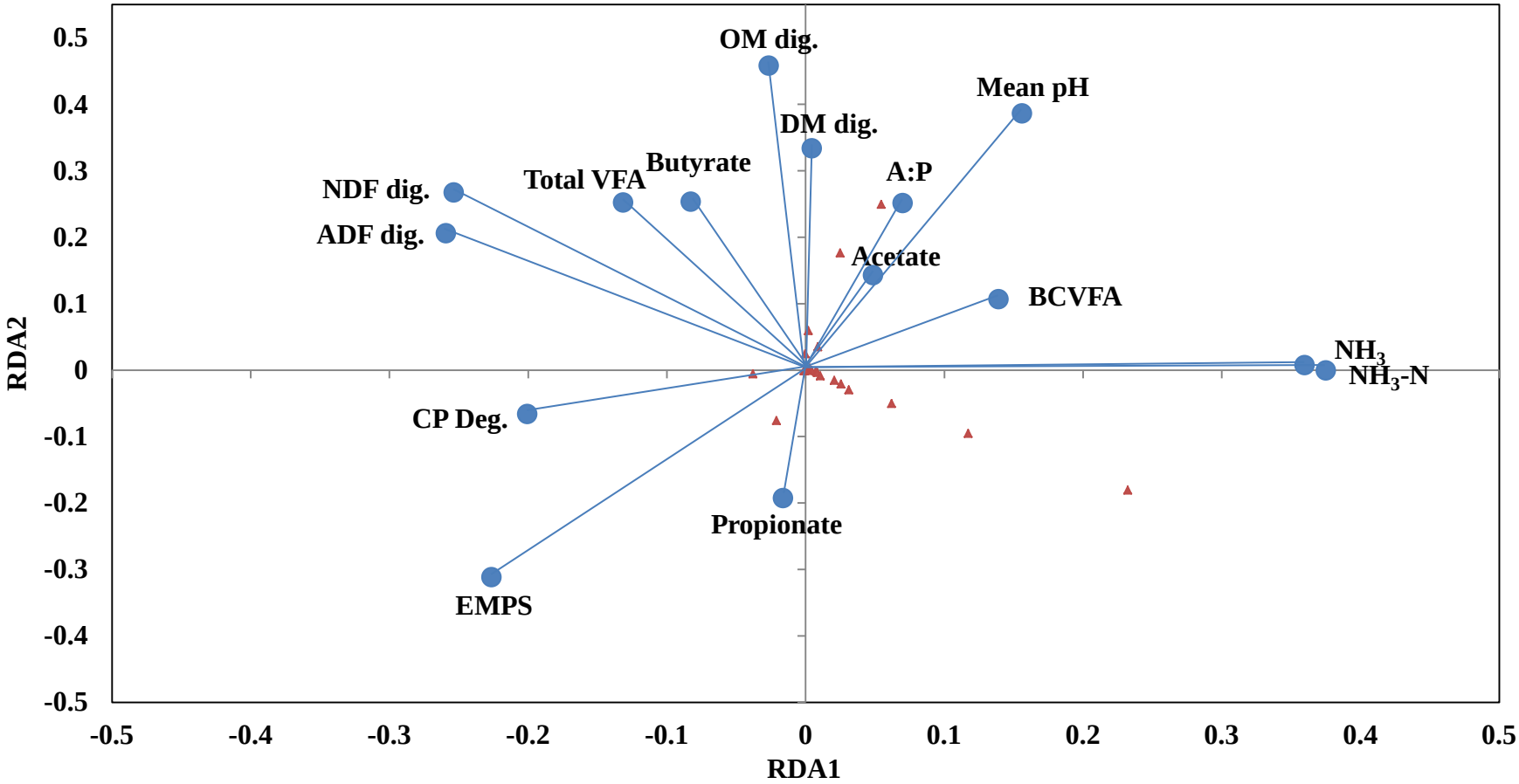
[1]Level 2 KEGG Orthology terms as predicted by PICRUSt.
[2]Data is presented as means ± standard error of the mean with n=4 samples per type.
[3]Probabilty corresponding to the null hypothesis.
[4]Data is presented as means ± standard error of the mean with n=48 samples per type.
[5]Sample types with different superscripts in a row are different at $P < 0.05$.

Table 3.12. Differences in level 2 PICRUSt functional inferences between sample types (*continued from pg. 116).*

Item[1]	Sample Type			P-value[4]
	Rumen[2]	Inocula[2]	Fermenter[3]	
Biosynthesis of other secondary metabolites[3]	1.06 ± 0.03[a]	1.07 ± 0.01[a]	0.98 ± 0.01[b]	0.002
Cell growth and death	0.61 ± 0.01	0.61 ± 0.01	0.59 ± 0.00	0.009
Infectious diseases	0.40 ± 0.01	0.40 ± 0.01	0.41 ± 0.00	0.278
Endocrine system	0.35 ± 0.01	0.36 ± 0.01	0.34 ± 0.00	0.708
Transport and catabolism[3]	0.35 ± 0.01[a]	0.34 ± 0.01[a]	0.30 ± 0.01[b]	0.004
Signaling molecules and interaction	0.17 ± 0.01	0.18 ± 0.00	0.17 ± 0.00	0.092
Environmental adaptation[3]	0.14 ± 0.00[ab]	0.14 ± 0.00[a]	0.15 ± 0.00[b]	<0.001
Metabolic diseases[3]	0.12 ± 0.00[a]	0.12 ± 0.00[a]	0.12 ± 0.00[b]	0.002
Cancers[3]	0.11 ± 0.00[ab]	0.11 ± 0.00[a]	0.12 ± 0.00[b]	0.001
Nervous system[3]	0.10 ±0.00[ab]	0.11 ± 0.00[a]	0.10 ± 0.00[b]	0.002
Neurodegenerative diseases	0.10 ± 0.00	0.10 ± 0.00	0.09 ± 0.00	0.632
Immune system	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.619
Digestive system	0.09 ± 0.01	0.10 ± 0.01	0.10 ± 0.00	0.708
Immune system diseases	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.138
Excretory system[3]	0.02 ± 0.00[a]	0.02 ± 0.00[ab]	0.01 ± 0.00[b]	0.004

[1]Level 2 KEGG Orthology terms as predicted by PICRUSt.
[2]Data is presented as means ± standard error of the mean with n=4 samples per type.
[3]Probabilty corresponding to the null hypothesis.
[4]Data is presented as means ± standard error of the mean with n=48 samples per type.
[5]Sample types with different superscripts in a row are different at $P < 0.05$.

Figure 3.1. Redundancy analysis plot of fermentation parameters based on microbial ecology data.[1]



[1]Key: OM dig.= true organic matter digestion (%), DM dig. =true dry matter digestion (%), NDF dig. = neutral detergent fiber digestion (%), ADF dig. = acid detergent fiber digestion (%), CP deg = crude protein degradation (%) Total VFA= total volatile fatty acid concentration (m*M*), Acetate= acetate concentration (mol/100 mol), Propionate= concentration (mol/100 mol), BCVFA = branched-chain volatile fatty acid concentration (m*M*), A:P= acetate:propionate ratio, NH_3= ammonia concentration (mg/dl), NH_3-N = Ammonia nitrogen flow(g/d), EMPS= Efficiency of microbial protein synthesis (g of microbial N/kg of OM truly digested)

Figure 3.2. Principal component analysis plot of sample type and trial day.

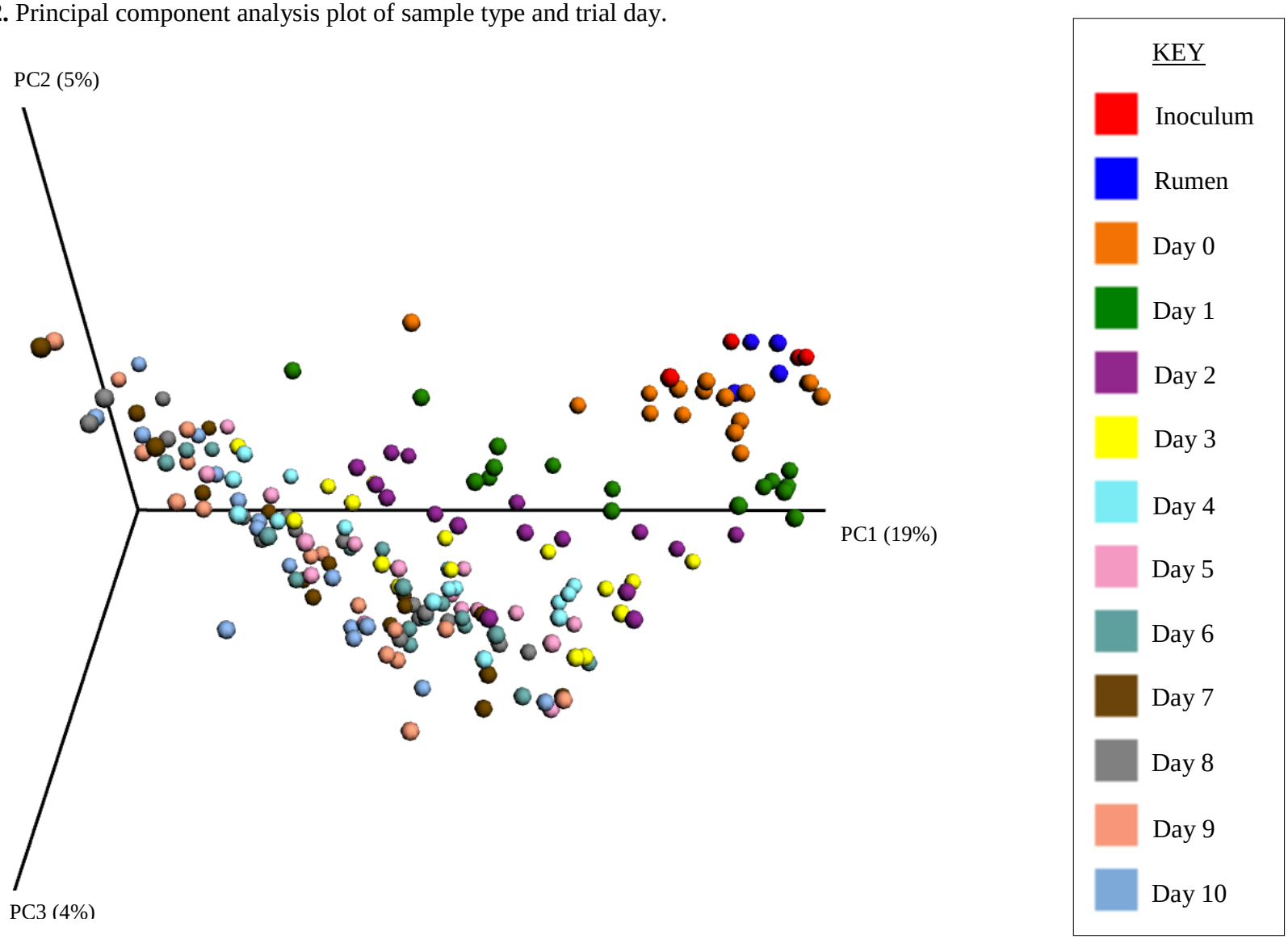
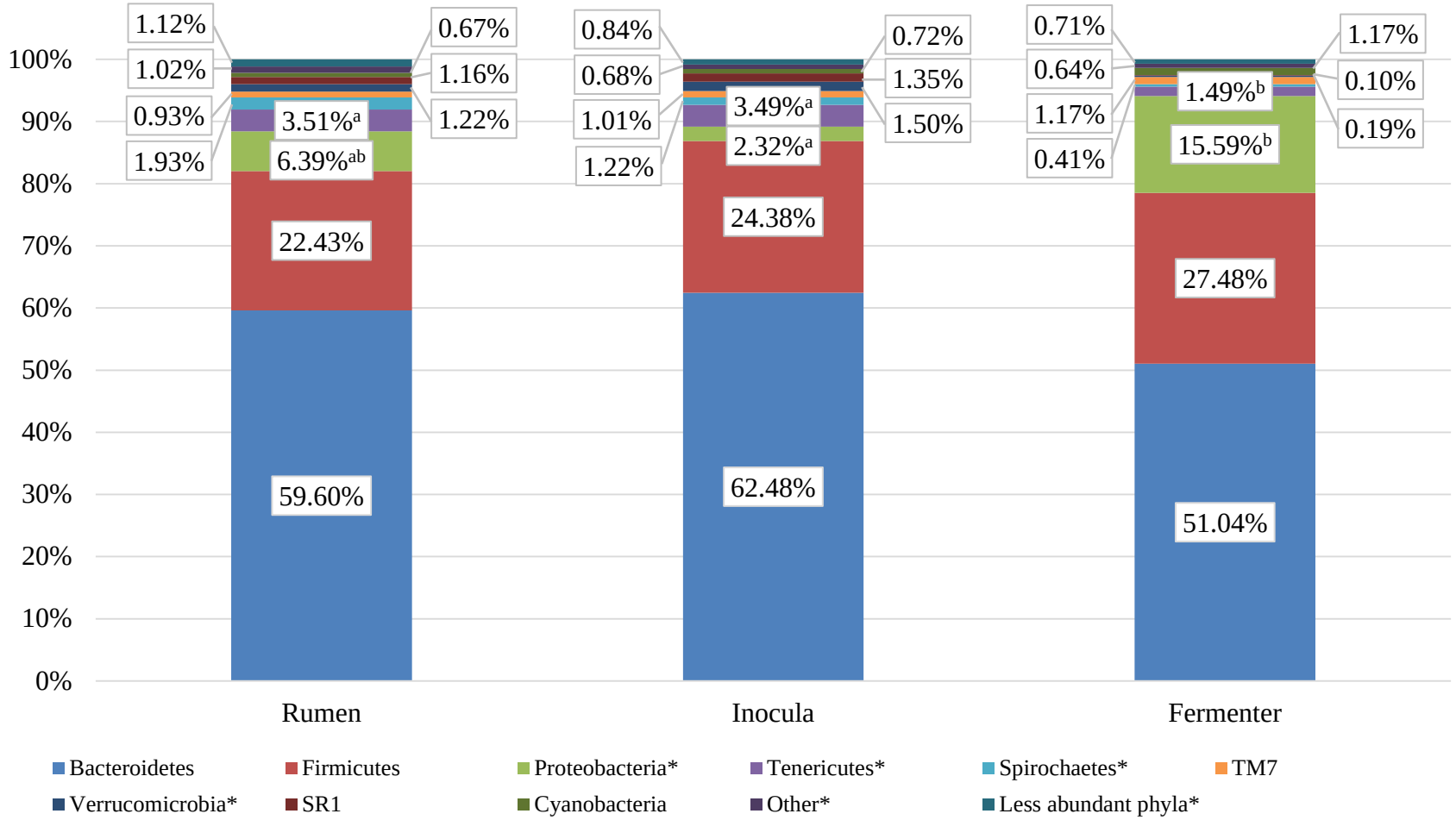
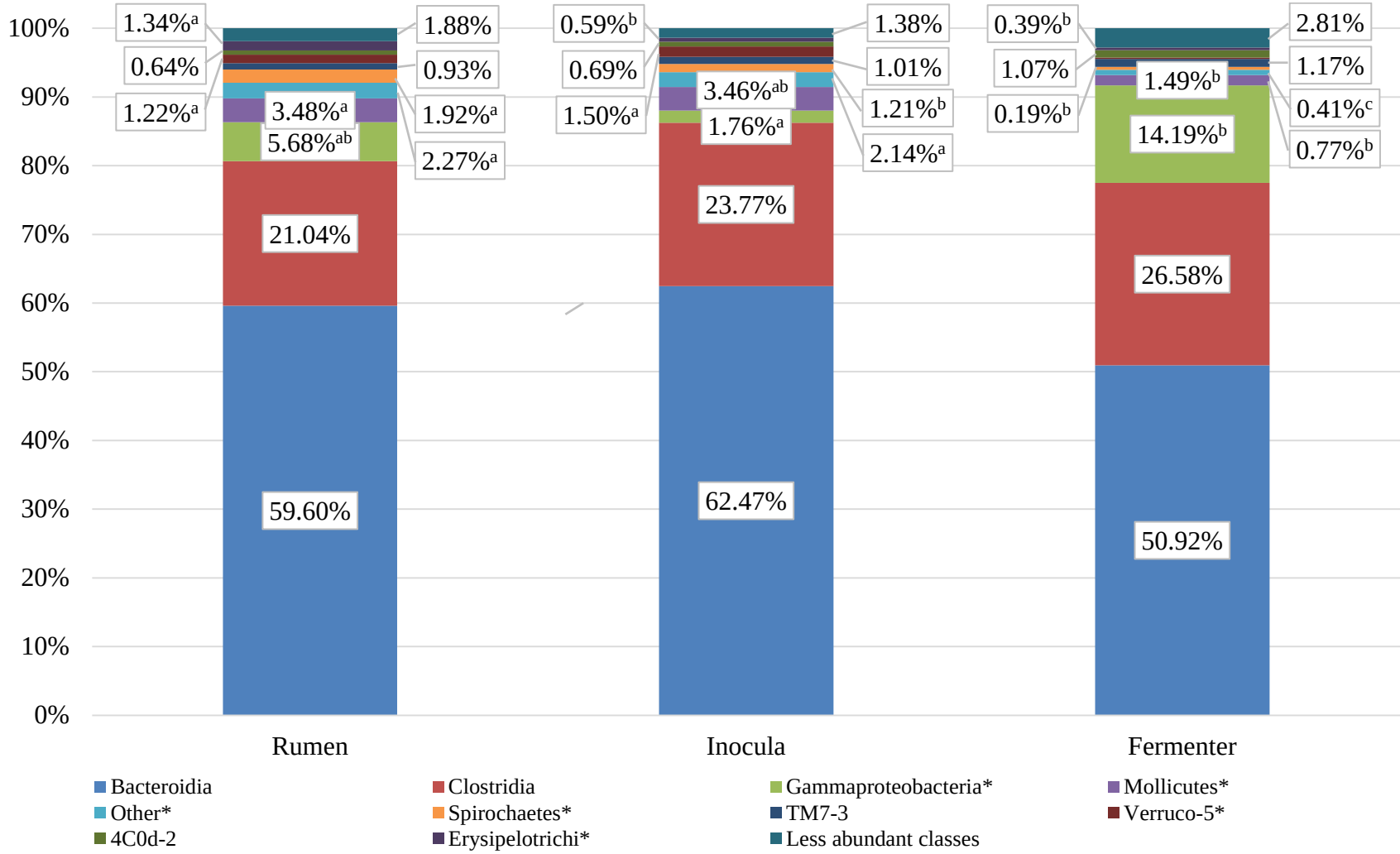


Figure 3.3. Relative abundance of phyla within rumen, inoculum, and fermenter samples.[1, 2]



[1]Phyla denoted with an asterisk (*) differ ($P < 0.05$) between sample types.
[2]Differing superscripts denotes that relative abundance varies ($P < 0.05$) by sample type within the designated phylum.

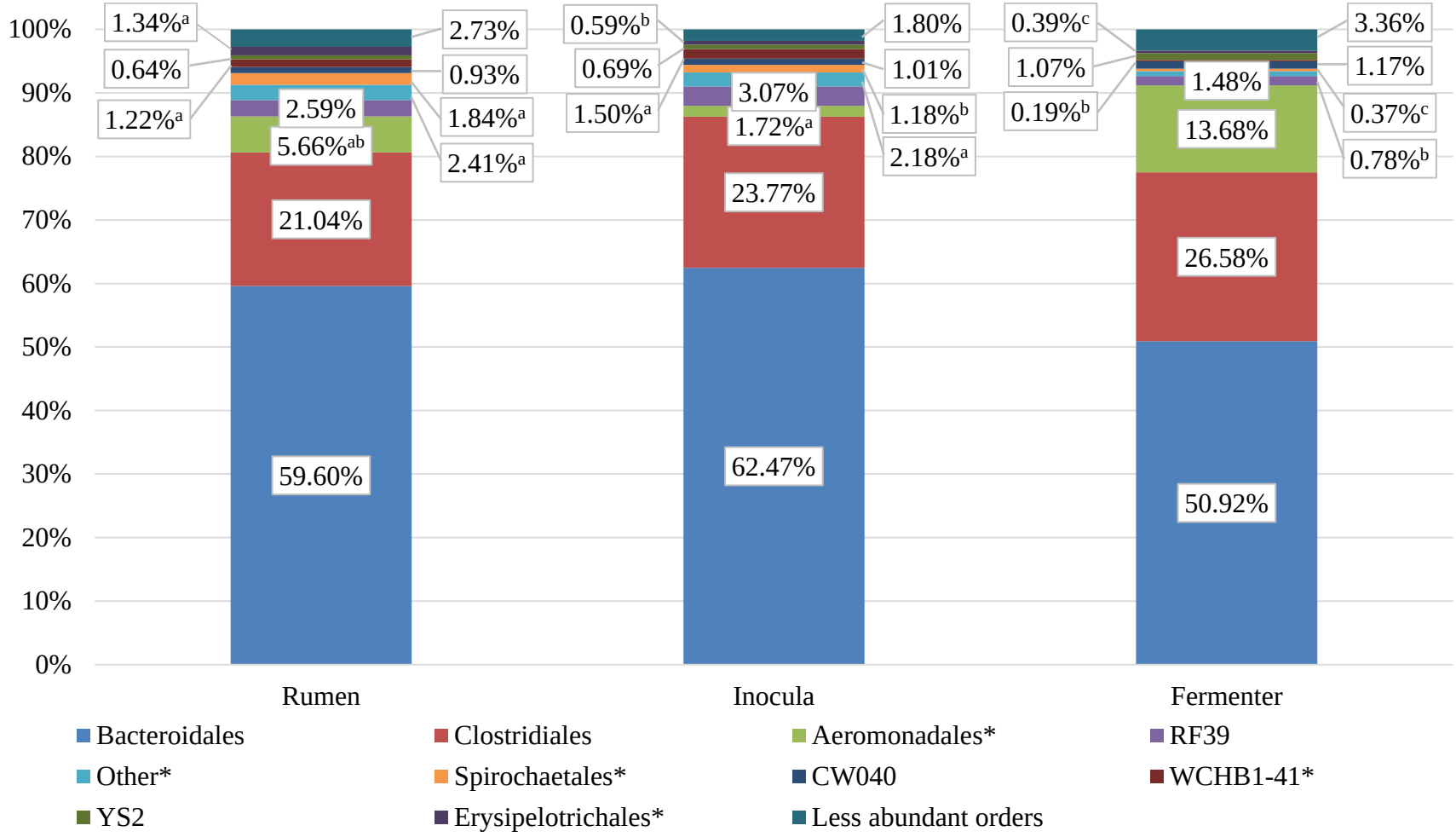
Figure 3.4. Relative abundance of classes within rumen, inoculum, and fermenter samples.[1, 2]



[1]Class denoted with an asterisk (*) differ ($P < 0.05$) between sample types.

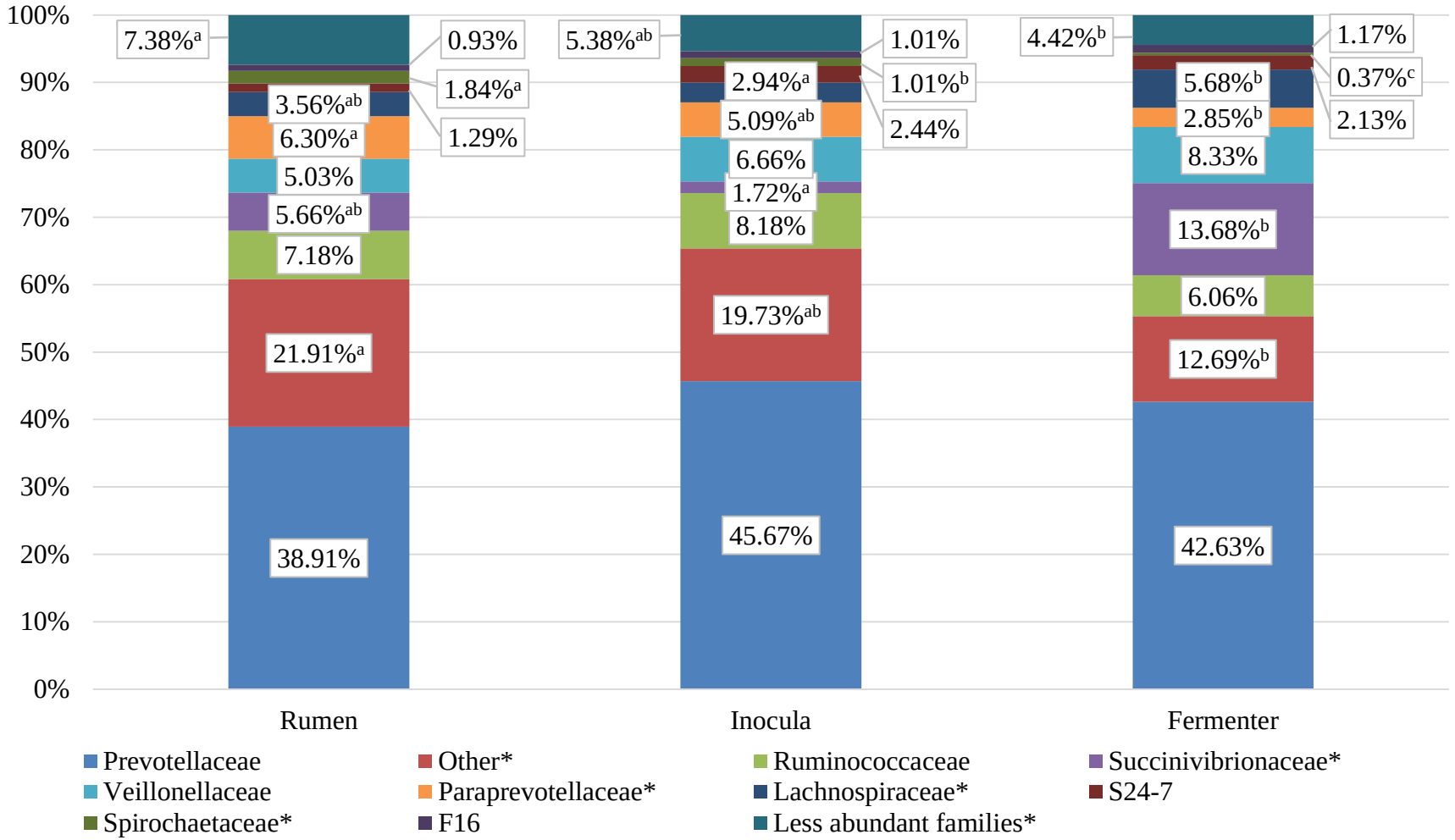
[2]Differing superscripts denotes that relative abundance varies ($P < 0.05$) by sample type within the designated class.

Figure 3.5. Relative abundance of orders within rumen, inoculum, and fermenter samples.[1, 2]



[1]Families denoted with an asterisk (*) differ ($P < 0.05$) between sample types.
[2]Differing superscripts denote that relative abundance varies ($P < 0.05$) by sample type within the designated family.

Figure 3.6. Relative abundance of families within rumen, inoculum, and fermenter samples.[1, 2]



[1]Families denoted with an asterisk (*) differ ($P < 0.05$) between sample types.

[2]Differing superscripts denote that relative abundance varies ($P < 0.05$) by sample type within the designated family.

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APPENDIX

Additional Data from Experiment 2

Table A1. Differences in phyla composition between sample types.

Phyla	Sample Type			F-Value	P-Value[3]
	Rumen[1]	Inocula[1]	Fermenter[2]		
Bacteriodetes	59.60 ± 4.50	62.48 ± 5.68	51.04 ± 2.06	1.80	0.176
Firmicutes	22.43 ± 2.69	24.38 ± 2.77	27.48 ± 1.21	0.95	0.393
Proteobacteria	6.39 ± 3.47^{ab}	2.32 ± 1.00^{a}	15.59 ± 1.30^{b}	5.74	<0.001
Tenericutes	3.51 ± 0.92^{a}	3.49 ± 0.98^{a}	1.49 ± 0.23^{b}	5.56	0.007
Spirochaetes	0.93 ± 0.32^{a}	1.01 ± 0.32^{b}	1.17 ± 0.17^{c}	38.28	<0.001
TM7	0.67 ± 0.28	0.72 ± 0.16	1.17 ± 0.13	0.11	0.900
Verrucomicrobia	1.02 ± 0.18^{a}	0.68 ± 0.08^{a}	0.64 ± 0.04^{b}	87.38	<0.001
Cyanobacteria	1.93 ± 0.52	1.22 ± 0.39	0.41 ± 0.04	1.16	0.323
Other	1.22 ± 0.53^{a}	1.50 ± 0.29^{ab}	0.19 ± 0.03^{b}	3.64	0.033
Less abundant phyla[4]	2.28 ± 0.51^{a}	2.19 ± 0.81^{a}	0.81 ± 0.06^{b}	28.63	<0.001

[1]Data presented as means ± standard error of the mean with n=4 samples per type.
[2]Data presented as means ± standard error of the mean with n=48 samples per type.
[3]Probability corresponding to the null hypothesis.
[4]Phyla present at less than 0.75% of total OTUs.

Table A2. Differences in class composition between sample types

Class	Sample Type			F-Value	P-Value[3]
	Rumen[1]	Inoculum[1]	Fermenter[2]		
Bacteroidia	59.60 ± 4.50	62.47 ± 5.68	50.92 ± 2.06	1.84	0.170
Clostridia	21.04 ± 2.51	23.77 ± 2.65	26.58 ± 1.18	1.12	0.333
Gammaproteobacteria	5.68 ± 3.52^{ab}	1.76 ± 0.96^{b}	14.19 ± 1.21^{a}	5.74	0.006
Mollicutes	3.48 ± 0.92^{a}	3.46 ± 0.97^{ab}	1.49 ± 0.23^{b}	5.40	0.008
Other	0.93 ± 0.32^{a}	1.01 ± 0.32^{a}	1.17 ± 0.17^{b}	42.16	<0.001
Spirochaetes	0.64 ± 0.28^{a}	0.69 ± 0.17^{b}	1.07 ± 0.12^{c}	37.70	<0.001
TM7-3	2.27 ± 0.40	2.14 ± 0.70	0.77 ± 0.05	0.11	0.900
Verruco-5	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.71 ± 0.07^{b}	87.23	<0.001
4C0d-2	1.92 ± 0.52	1.21 ± 0.39	0.41 ± 0.04	0.88	0.423
Erysipelotrichi	1.34 ± 0.56^{a}	0.59 ± 0.17^{b}	0.39 ± 0.03^{b}	18.46	<0.001
Betaproteobacteria	1.22 ± 0.53^{a}	1.50 ± 0.28^{a}	0.19 ± 0.03^{b}	8.07	0.001
Less abundant classes[4]	1.46 ± 0.31	0.78 ± 0.19	1.04 ± 0.08	1.33	0.274

[1]Data presented as means ± standard error of the mean with n=4 samples per type.
[2]Data presented as means ± standard error of the mean with n=48 samples per type.
[3]Probability corresponding to the null hypothesis.
[4]Classes present at less than 0.75% of total OTUs.

Table A3. Differences in order composition between sample types.

Order	Sample Type			F-Value	P-Value[3]
	Rumen[1]	Inoculum[1]	Fermenter[2]		
Bacteroidales	59.60 ± 4.50	62.47 ± 5.68	50.92 ± 2.06	1.84	0.170
Clostridiales	21.04 ± 2.51	23.77 ± 2.65	26.58 ± 1.18	1.12	0.333
Aeromonadales	5.66 ± 3.51^{ab}	1.72 ± 0.95^{a}	13.68 ± 1.20^{b}	5.33	0.008
RF39	2.59 ± 0.47	3.07 ± 0.86	1.48 ± 0.23	6.70	<0.001
CW040	0.93 ± 0.32	1.01 ± 0.32	1.17 ± 0.17	0.11	0.900
YS2	0.64 ± 0.28	0.69 ± 0.17	1.07 ± 0.12	0.88	0.423
Other	2.41 ± 0.46^{a}	2.18 ± 0.70^{a}	0.78 ± 0.06^{b}	44.31	<0.001
Burkholderiales	0.02 ± 0.01^{a}	0.01 ± 0.00^{a}	0.71 ± 0.07^{b}	8.13	0.001
Spirochaetales	1.84 ± 0.53^{a}	1.18 ± 0.38^{b}	0.37 ± 0.04^{c}	39.71	<0.001
Erysipelotrichales	1.34 ± 0.56^{a}	0.59 ± 0.17^{b}	0.39 ± 0.03^{b}	18.46	<0.001
Lactobacillales	0.05 ± 0.02	0.02 ± 0.01	0.48 ± 0.11	1.31	0.280
WCHB1-41	1.22 ± 0.53^{a}	1.50 ± 0.28^{a}	0.19 ± 0.03^{b}	87.23	<0.001
Desulfovibrionales	0.06 ± 0.03	0.09 ± 0.04	0.30 ± 0.03	4.29	0.019
Methanobacteriales	0.09 ± 0.02	0.20 ± 0.09	0.24 ± 0.03	1.08	0.346
Coriobacteriales	0.03 ± 0.02^{a}	0.03 ± 0.01^{a}	0.23 ± 0.02^{b}	10.56	<0.001
Less abundant orders[4]	2.48 ± 0.65	1.46 ± 0.42	1.40 ± 0.14	2.25	0.116

[1]Data presented as means ± standard error of the mean with n=4 samples per type.
[2]Data presented as means ± standard error of the mean with n=48 samples per type.
[3]Probability corresponding to the null hypothesis.
[4]Orders present at less than 0.10% of total OTUs.

Table A4. Differences in family composition between sample types.

Family	Sample Type			F-Value	P-Value[3]
	Rumen[1]	Inoculum[1]	Fermenter[2]		
Prevotellaceae	38.91 ± 4.62	45.67 ± 8.77	42.63 ± 2.08	0.23	0.797
Other	21.91 ± 2.89[a]	19.73 ± 4.36[ab]	12.69 ± 0.95[b]	5.42	0.007
Ruminococcaceae	7.18 ± 0.48	8.18 ± 1.06	6.06 ± 0.76	0.39	0.677
Succinivibrionaceae	5.66 ± 3.51[ab]	1.73 ± 0.95[a]	13.68 ± 1.20[b]	5.33	0.008
Veillonellaceae	5.03 ± 1.49	6.66 ± 0.78	8.33 ± 0.69	1.11	0.337
Paraprevotellaceae	6.31 ± 1.11[a]	5.09 ± 1.09[ab]	2.85 ± 0.28[b]	7.98	0.001
Lachnospiraceae	3.56 ± 0.43[ab]	2.94 ± 0.32[a]	5.68 ± 0.31[b]	4.98	0.011
S24-7	1.29 ± 0.37	2.44 ± 0.58	2.12 ± 0.28	0.45	0.639
F16	0.93 ± 0.32	1.01 ± 0.32	1.17 ± 0.17	0.11	0.900
RF16	1.40 ± 0.29[a]	1.12 ± 0.14[a]	0.28 ± 0.04[b]	33.14	<0.001
Spirochaetaceae	1.06 ± 0.53[a]	1.19 ± 0.38[b]	0.37 ± 0.04[c]	39.71	<0.001
Erysipelotrichaceae	1.34 ± 1.34[a]	0.59 ± 0.17[b]	0.39 ± 0.03[b]	18.46	<0.001
RFP12	0.88 ± 0.35[a]	1.10 ± 0.12[a]	0.16 ± 0.03[b]	69.22	<0.001
Anaeroplasmataceae	0.81 ± 0.44	0.37 ± 0.12	0.01 ± 0.00	0.30	0.762
Comamonadaceae	0.01 ± 0.00[a]	0.01 ± 0.00[a]	0.68 ± 0.07[b]	7.67	0.001
Clostridiaceae	0.28 ± 0.06[a]	0.21 ± 0.02[ab]	0.14 ± 0.02[b]	4.01	0.024
WCHB1-25	0.29 ± 0.15[a]	0.30 ± 0.14[a]	0.04 ± 0.01[b]	96.04	<0.001
p_2534_18B5	0.40 ± 0.12[a]	0.11 ± 0.04[b]	0.04 ± 0.01[b]	24.29	<0.001
Lactobacillaceae	0.04 ± 0.02	0.02 ± 0.01	0.47 ± 0.11	1.27	0.291
Methanobacteriaceae	0.09 ± 0.02	0.20 ± 0.09	0.24 ± 0.03	1.08	0.346
Mogibacteriaceae	0.19 ± 0.03	0.21 ± 0.04	0.12 ± 0.01	2.19	0.122

[1]Data presented as means ± standard error of the mean with n=4 samples per type.

[2]Data presented as means ± standard error of the mean with n=48 samples per type.

[3]Probability corresponding to the null hypothesis.

[4]Families present at less than 0.10% of total OUT/

Table A4. Differences in family composition between sample types (*continued from pg. 157).*

Family	Sample Type			F-Value	P-Value[3]
	Rumen[1]	Inoculum[1]	Fermenter[2]		
Christensenellaceae	0.19 ± 0.05[a]	0.28 ± 0.08[b]	0.03 ± 0.01[c]	60.76	<0.001
Elusimicrobiaceae	0.21 ± 0.09	0.14 ± 0.02	0.12 ± 0.03	0.55	0.582
Desulfovibrionaceae	0.06 ± 0.03	0.08 ± 0.04	0.30 ± 0.03	4.29	0.019
Fibrobacteraceae	0.33 ± 0.10[a]	0.08 ± 0.03[b]	0.02 ± 0.01[b]	57.49	<0.001
Bacteroidaceae	0.15 ± 0.03	0.06 ± 0.02	0.08 ± 0.02	0.59	0.560
Coriobacteriaceae	0.03 ± 0.02[a]	0.03 ± 0.01[a]	0.23 ± 0.02[b]	6.00	0.005
Less abundant families[4]	0.70 ± 0.13	0.48 ± 0.12	1.08 ± 0.12	1.37	0.265

[1]Data presented as means ± standard error of the mean with n=4 samples per type.
[2]Data presented as means ± standard error of the mean with n=48 samples per type.
[3]Probability corresponding to the null hypothesis.
[4]Families present at less than 0.10% of total OUT

Figure A1. Relative abundance of families within rumen, inoculum, and fermenter operation

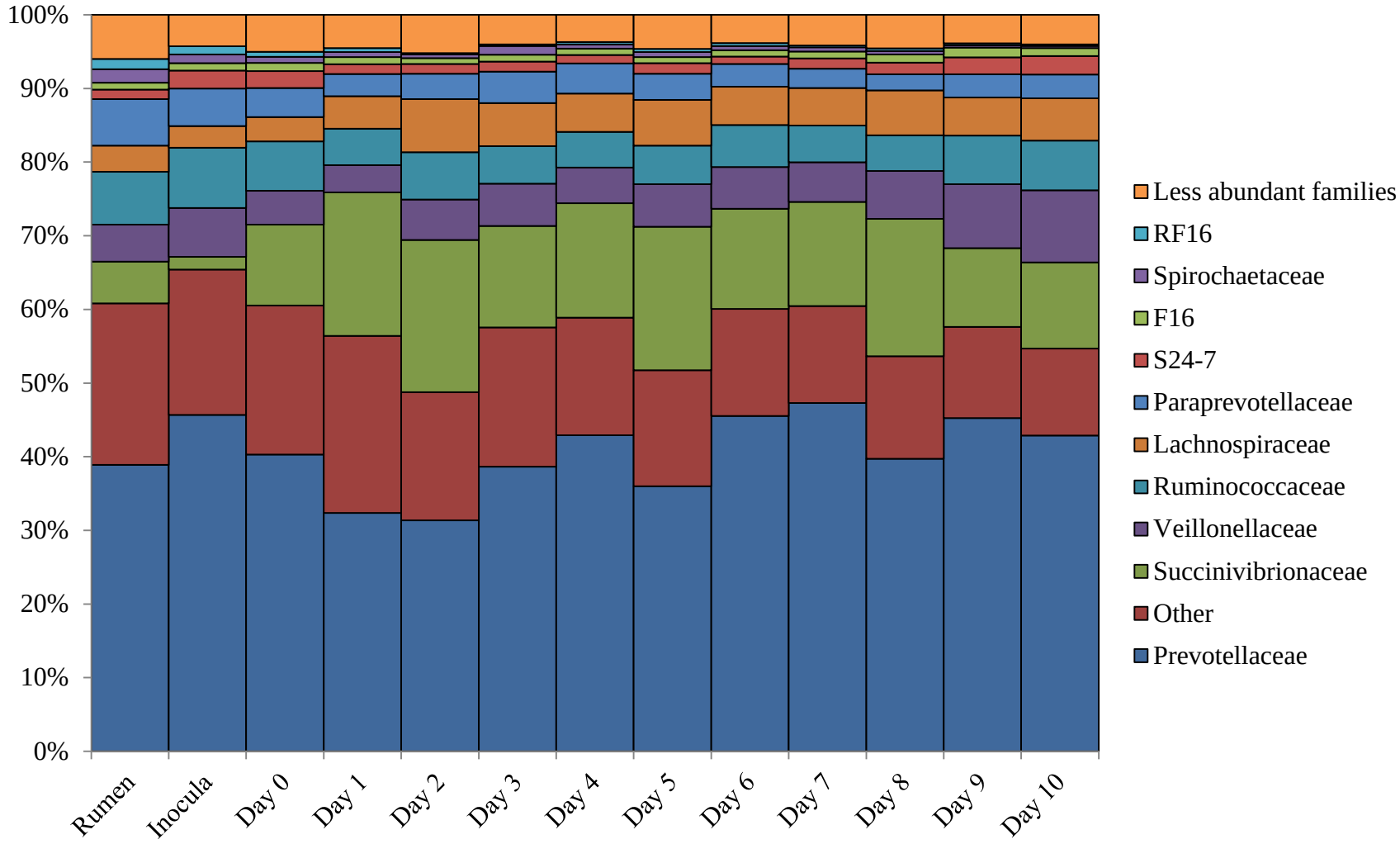

Figure A2. Relative abundance of families designated as 'less abundant' in figure A1 within samples from the rumen, inoculum, and each day of fermenter operation.